RESEARCH ARTICLE

Immunohistopathological Study of c-FLIP Protein in Mycosis Fungoides

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Abstract

Background: Mycosis fungoides (MF) is the commonest variant of primary cutaneous T cell lymphoma with several clinicopathologic variants. Defective apoptotic mechanism may be important in the pathogenesis and progression of MF. c-FLIP protein is an important anti-apoptotic marker and chemotherapeutic resistant factor. This study aimed to evaluate the c-FLIP expression in MF and its role in the pathogenesis of MF. **Methods:** Twenty patients of MF and ten normal persons were included in this study. Skin biopsies were obtained from both patients and controls. They were studied and examined immunohistochemically for the expression of CD4 and c-FLIP. **Results:** c-FLIP expression was significantly increased in patients when compared to controls in both epidermis and dermis. There were positive correlations between c-FLIP expression and CD4+ expression in both epidermal and dermal lesions of patients group. There were statistically significant positive correlations between c-FLIP expression (in both dermal and epidermal lesions) and the age of patients. c-FLIP expression increased with the tumor progression but with no statistical significance. **Conclusion:** Defective regulation of apoptosis has been considered as a main cause for accumulation of clonal T cells, and it was related to an increased expression of c-FLIP which may have a role in the pathogenesis of MF. Also, c-FLIP may have prognostic information in MF as its level increased with both age of the patients and tumor progression.

Keywords: c-FLIP protein- mycosis fungoides- pathogenesis

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Introduction

Cutaneous T- cell lymphoma (CTCL) represents a group of diseases that are heterogeneous in clinical diagnosis and presentation. The incidence of CTCL is 6.4 per million and it is rising making it the second most common extra nodal non Hodgkin lymphoma with primary lesion in skin. It is described as clonal proliferation of malignant CD4 skin-homing lymphocytes. The most common variants are mycosis fungoides (MF) and Sezary syndrome (Willemze et al., 2005). The classical type of MF has 4 stages: patch, plaque, tumor and erythroderma. However many clinical and histopathological variants having atypical clinical presentations were described such as hypopigmented and poikilodermatic (Huang et al., 2014). At present it is difficult to accurately diagnose MF. The current approach is to combine clinicopathological features in three categories; clinical presentations, pathologic features, and presence of T cell receptor (TCR) gene clonal rearrangements (International Society of Cutaneous Lymphomas ISCL Criteria, 2005) (Pimpinelli et al., 2005).

Apoptosis or programmed cell death is critical for tissue homeostasis. Normally, in healthy skin the proliferation of cells in the basal cell layer is balanced and regulated by keratinocytes in the superficial layer of epidermis through the process of apoptosis (Plakowska et al., 1994). Keratinocytes may undergo apoptosis by loss of cell-cell contact after crosslinking of the Fas molecule (Kruger et al., 2001).

A key inhibitor of death receptor signaling is cellular FLICE inhibitory protein (c-FLIP). It interacts with Fas-associated death domain protein (FADD) and procaspase-8 to inhibit the initiation of apoptotic cascade (Irmler et al., 1997). Defective regulation of apoptosis has been considered as a main cause for accumulation of clonal T cells (Zhang et al., 2003). It was related to an impaired expression of Fas and c-FLIP which may have a role in the pathogenesis of MF (Contassot et al., 2008). So the aim of this study was to evaluate the expression of c-FLIP in MF and its role in the pathogenesis of the disease.

Materials and Methods

After approval of the research ethics committee of Tanta Faculty of Medicine (approval code 2911/12/14), this study was a case-control study conducted on 20 patients with MF, recruited from the Out-Patient Clinic of Dermatology and Venereology Department, Tanta University Hospitals during the period from December

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2014 to December 2015. Inclusion criteria were newly diagnosed cases and patients who stopped topical treatment for 1 week or systemic treatment for at least 6 weeks. Patients with autoimmune and hyper proliferative diseases as psoriasis, patients under phototherapy or photochemotherapy, pregnant and lactating female or who were under hormonal therapy were excluded from the study. The study included 10 normal persons who served as a control group.

All patients were subjected to detailed history taking (age, sex, duration of lesions and distribution of lesions), thorough general and dermatological examination to determine the clinical variant, and assessment of the diseases severity (staging). Routine investigations were done such as: Complete blood picture, fasting and postprandial blood glucose levels, hepatic and renal function tests and plain chest X-ray and pelvi-abdominal ultrasonography. Written consents were obtained from all participants in the study. All patients were photographed at first clinical presentation.

Skin biopsies (punch 4 mm) were taken, formalin-fixed, routinely processed. Paraffin-embedded tissue sections (3-5µ) were prepared on charged glass slides for: routine H&E staining for confirmation of clinical diagnosis of MF and immunohistochemical staining of sections using antibodies against CD4 (Mouse monoclonal antibody, Dako UK Ltd, Ely, UK) and c-FLIP (Rabbit monoclonal antibody, Gene Bank no. gil 12643547, Novusbio).

Slides were de-paraffinized in xylene and rehydrated through a graded alcohol series before being placed in 3% hydrogen peroxide (H_2O_2) /methanol blocking solution to quench endogenous peroxidase activity, followed by subsequent antigen unmasking. Incubation with the primary antibodies was performed for 30 minutes at room temperature at the following dilutions: 1:20 for CD4 antibodies and 1:3000 for c-FLIP antibodies

After washing with phosphate buffer saline (PBS), the slides were incubated for 30 min at room temperature with anti-mouse or anti-rabbit universal immunostaining IgG (biotin-conjugated secondary antibody) conjugated to a streptavidin peroxidase-labeled polymer (Neo Markers Biotechnology). Reactions were developed with 3, 3'-diaminobenzidine chromogen and counterstained with hematoxyline for 10 seconds, then washed with several changes of deionized water. Sections were dehydrated through alcohol 95%, then through xylene. Excess xylene was then wiped off and 1-2 drops of permanent mounting media and a glass cover-slip were applied.

Evaluation of the immune-stained sections (Stutz et al., 2012)

CD4 expression was detected as homogenous brown stain in the cell membrane of corresponding lymphocytes. For CD4, the immune-reactive cells were scored as mean number of positive cells per high- power fields (×400) of the entire length of epidermis and dermis (number of positive cells per 10 non- overlapping high- power fields HPF). So, density score of CD4 was as follows: 0= 1-10, +1= 11-25 (mild), +2= 26-50 (moderate), +3= greater than 50 (strong)

c-FLIP expression was detected as homogenous brown stain in the cytoplasm of lymphocytes and keratinocytes. The c- FLIP intensity score was classified from zero (no staining), 1: mild, 2: moderate, 3: strong staining. The mean percentage of positive cells was determined in at least ten areas randomly at a magnification of ×400 (HPF) and the proportion of stained cells were assessed as: 0= 0-10%, +1= 11-25% (mild), +2= 26-50% (moderate), +3= greater than 50% (strong). Assessment of the staining was evaluated by two independent pathologists. The score of the proportion of stained cells for each field was multiplied by the score of the staining intensity of that field to provide an immunostaining intensity– distribution index (IIDI) (Abdel-Latif et al., 2009, Ryang et al. 2007).

Statistical analysis of the data: Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 (v 16; SPSS Inc., Chicago, IL, USA).

Results

The patients in this study included 12 females (60%) and 8 males (40%), their ages ranged from 6 to 60 years with a mean of 39.90 ± 16.63 years. The control group included 6 females (60%) and 4 males (40%), their ages ranged from 12 to 50 years with a mean of 35.30 ± 10.69 years. There were no statistically significant differences between the age and sex of both groups. The duration of the disease ranged from 0.08 to 8 years with a mean of 2.48 ± 2.38 years.

Clinically MF patients were classified as: 10 patients of classic MF (50%) further subdivided into 9 with patch stage (45%) and 1 with plaque stage (5%). The remaining 10 cases (50%) were subdivided into: 6 hypopigmented (30%), 2 poikilodermatic (10%), 1 erythrodermic (5%), and 1 hyperpigmented (5%). According to ISCL/ EORTC, MF patients were classified as follow: 9 patients of stage IA (45%), 10 patients of stage IB (50%), 1 patient of stage IIIA (5%). None of the patients included in the study had lymphadenopathy or organomegaly.

CD4 density score in the patients ranged from 0 to +3 with a mean of (1.75 ± 0.91) in the epidermal lesions,

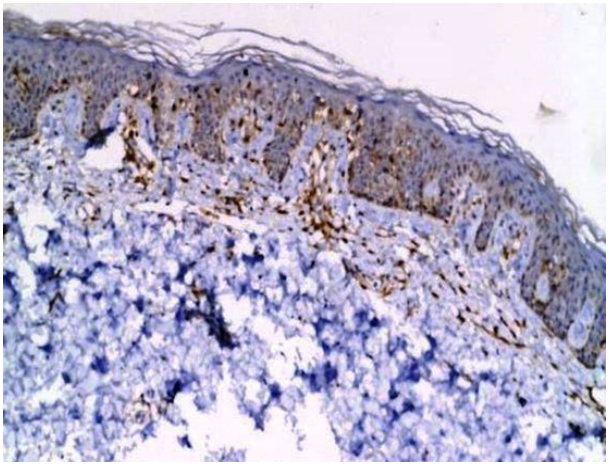

Figure 1. CD4 Immunostaining of Patchy Classic MF Section Showing Moderate (+2) Stained Epidermotropic Lymphocytes and Discrete Superficial Perivascular Positive Dermal Infiltrate (PAP X 200).

Table 1. Comparison Between The Studied Groups (Patients and Controls) According to CD4 Density Score and C-FLIP Intensity Score

CD4 density score	Patients (n=20) No.	%	Control (n=10) No.	%	c-FLIP intensity score	Patients (n=20) No.	%	Control (n=10) No	%
Epidermis					Epidermis				
0	1	5	6	60	0	0	0	6	60
1+ (mild)	8	40	4	40	1+ (mild)	8	40	4	40
2+ (moderate)	6	30	0	0	2+ (moderate)	6	30	0	0
3+ (strong)	5	25	0	0	3+ (strong)	6	30	0	0
Min. – Max.	0.0 – 3.0		0.0 – 1.0		Min. – Max.	1.0 – 3.0		0.0 – 1.0	
Mean ± SD.	1.75 ± 0.91		0.40 ± 0.52		Mean ± SD.	1.90 ± 0.85		0.40 ± 0.52	
Median	2		0		Median	2		0	
Z (p)	3.548 (<0.001*)				Z (p)	3.868 (<0.001*)			
Dermis					Dermis				
0	4	20	8	80	0	8	40	10	100
1+ (mild)	10	50	2	20	1+ (mild)	12	60	0	0
2+ (moderate)	5	30	0	0	2+ (moderate)	0	0	0	0
3+ (strong)	1	0	0	0	3+ (strong)	0	0	0	0
Min. – Max.	0.0 – 2.0		0.0 – 1.0		Min. – Max.	0.0 – 1.0		0.0 – 0.0	
Mean ± SD.	1.10 ± 0.72		0.20 ± 0.42		Mean ± SD.	0.60 ± 0.50		0.0 ± 0.0	
Median	1		0		Median	1		0	
Z (p)	3.122 (0.002*)				Z (p)	3.109 (0.002*)			

Z, Z for Mann Whitney test; *, Statistically significant at p ≤ 0.05

while in the controls it ranged from 0 to +1 with a mean of (0.40 ± 0.52) and there was statistically significant difference between both groups with P-value (<0.001). In the dermal lesions of the patients, the score ranged from 0 to +3 with a mean of (1.10 ± 0.72), while in the control group it ranged from 0 to +1 with a mean of (0.20 ± 0.42) and there was statistically significant difference between the two groups with P-value (0.002). Table (1), Figures (1, 2)

c-FLIP intensity score in the patients ranged from 0 to +3 with a mean of 1.90 ± 0.85 in the epidermal lesions, while in the controls it ranged from 0 to +1 with a mean of 0.40 ± 0.52 and there was statistically significant difference between the two groups with P-value (<0.001). In the dermal lesions of the patients, it ranged from 0 to +1 with a mean of 0.60 ± 0.50 while in the controls it was 0 in all patients and there was statistically significant difference between the two groups with P-value (0.002). Table 1, Figures (3, 4, 5).

The immunostaining intensity– distribution index

Table 2. Comparison between the Studied Groups (Patients and Controls) According to c-FLIP Immunostaining Intensity– Distribution Index (IIDI)

c-FLIP immunostaining intensity– distribution index (IIDI)	Patients (n=20)	Control (n=10)
Epidermis		
Min. – Max.	1.0-9.0	0.0-1.0
Mean ± SD.	4.1± 0.58	0.62 ± 0.51
Median	6	1
Z (p)	4.346 (<0.001*)	
Dermis		
Min. – Max.	0.0 – 2.0	0.0 – 0.0
Mean ± SD.	0.75 ± 0.52	0.0 ± 0.0
Median	1	0
Z (p)	3.112 (0.002*)	

Z, Z for Mann Whitney test; *, Statistically significant at p ≤ 0.05

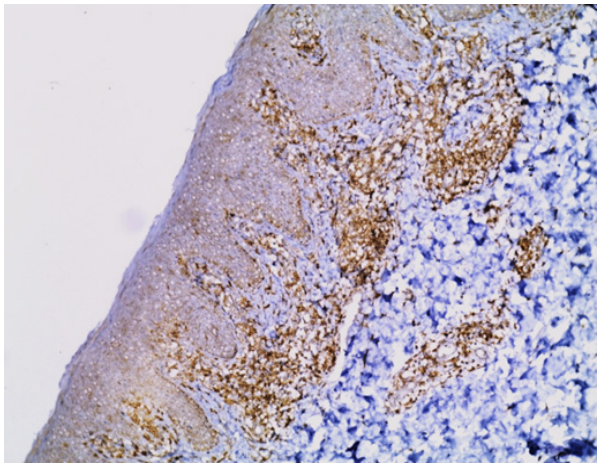

Figure 2. Plaque Stage Classic MF Section Showing CD4 Strong (+3) Immunostainig which Labels the Vast Majority of Epidermotropic Lymphocytes, Superficial and Deep Perivascular Dermal Lymphocytes (PAP X 200).

Table 3. Correlations between CD4 Density Score and C-FLIP Intensity Score with Age and Duration of Lesions in Patients Group

		CD4 density score		c-FLIP intensity score	
		Epidermis	Dermis	Epidermis	Dermis
Age (years)	rs	0.142	0.13	0.542	0.589
	p	0.551	0.586	0.014*	0.006*
Duration of disease (years)	rs	0.177	0.152	0.037	-0.027
	p	0.454	0.523	0.878	0.91
c-FLIP intensity score	rs	0.662	0.559		
	P	0.001*	0.010*		

r_s, Spearman coefficient; *, Statistically significant at $p \leq 0.05$

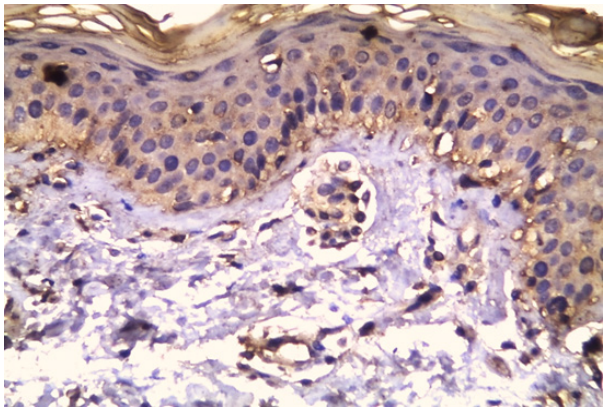

Figure 3. Patchy Stage of Classic MF Section Showing Moderate Staining (+2) of c-FLIP in Keratinocytes and Epidermotropic Lymphocytes with Mild (+1) Staining of Perivascular Lymphocytic Infiltrate (PAP × 400).

(IIDI) of c-FLIP in the patients ranged from 1 to 9 with a mean of 4.1± 0.58 in the epidermal lesions, while in the control group it ranged from 0 to 1 with a mean of 0.62 ± 0.51 and there was statistically significant difference between the two groups with P-value (<0.001). In the dermal lesions of the patients, IIDI ranged from 0 to 2 with a mean of 0.75 ± 0.52, while in the control group it was 0 in control group and there was statistically significant difference between the two groups with P-value (0.002). Table 2.

Regarding intensity of c-FLIP expression in different stages of MF, there were 9 patients of stage IA. They were

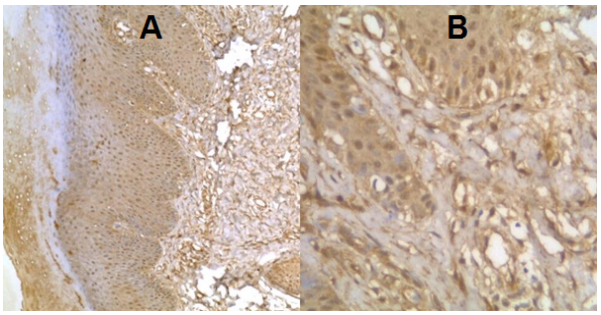

Figure 4. A, Plaque Stage MF Section Showing Strong Staining (+3) of c-FLIP in Keratinocytes and Epidermotropic Lymphocytes (PAP×200). B, Higher Magnification with (+1) Mild Staining of Dermal Lymphocytic Infiltrate (PAP×400)

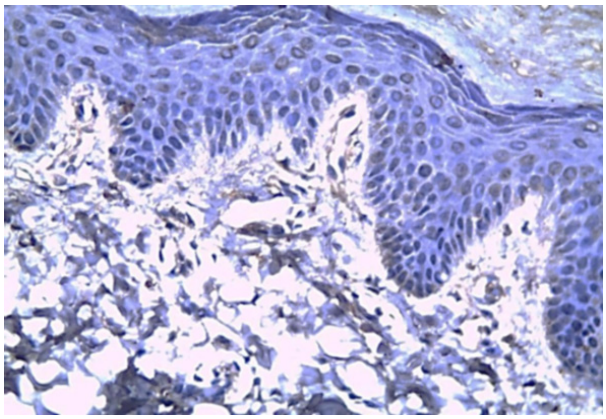

Figure 5. Section Showing Negative Staining of C-FLIP in Control Case. (PAP×400)

classified as: 6 patients (30%) with mild (+1) expression (IIDI ranged from 1-2), 2 patients (10%) with moderate (+2) expression (IIDI ranged from 2- 4), and 1 patient (5%) with strong (+3) expression (IIDI was 6). There were 10 patients of stage IB who were classified as: 2 patients (10%) with mild (+1) expression (IIDI ranged from 1-2), 4 patients (20%) with moderate (+2) expression (IIDI ranged from 3-6), 4 patients (20%) with strong (+3) expression (IIDI ranged from 6-9). One patient was of stage IIIA (5%). He was with strong (+3) expression (IIDI was 9). c-FLIP intensity score and IIDI increased with increasing stage of the tumor but with no statistical significance.

Regarding the correlations between CD4 and c-FLIP expression in both epidermis and dermis of the patients group, there were statistically significant positive correlations with P-value (0.001, 0.010) respectively. There were no statistically significant correlations between both age of patients and duration of lesions with CD4 density score. There was statistically significant positive correlation between c-FLIP intensity score (in both dermal and epidermal lesions) and the age of patients with P-values 0.006 and 0.014 respectively, while there was statistically insignificant correlation with the duration of lesions as shown in Table 3.

Discussion

Primary cutaneous lymphomas represent a heterogeneous group of T- and B-cell lymphomas that has resulted in controversy over diagnosis and classification in the past. MF is generally indolent in behavior and has a chronic course. Sezary syndrome is an aggressive and leukemic variant. Both conditions comprise approximately 53% of all cutaneous lymphomas, and are collectively referred to as cutaneous T-cell lymphomas (CTCLs) (Willemze et al., 2005).

Histologically, MF is characterized by a monoclonal proliferation of predominantly CD4+/CD45RO+ helper effector memory T cells. A low proliferation rate in early MF lesions, sparse apoptotic cells, and resistance to chemotherapeutic agents has led to the hypothesis that defects in apoptotic mechanisms may cause accumulation of atypical lymphocytes in the skin and are thus implicated

in the pathogenesis of MF (Willemze et al., 2005).

Apoptosis is a way to maintain cellular homeostasis and eliminate T cells that are no longer needed for an immune reaction. The death receptor or extrinsic pathway is an important apoptotic cascade in T cells that can be triggered by different ligands that bind to so-called death receptors which is trans-membrane proteins that belong to the family of tumor necrosis factors. Fas (CD95, Apo-1) is one of several death receptors that induces apoptosis through downstream activation of a caspase cascade after formation of a death-inducing signaling complex (DISC) induced by the binding of Fas ligand (Fas L) to cell surface Fas death receptors . Within the DISC, the Fas-associated death domain (FADD) protein cleaves and thereby activates caspase 8, which in turn activates caspase 3, leading to apoptosis (Stutz et al., 2012).

Cellular FLICE (FADD-like IL-1β-converting enzyme) inhibitory protein (c-FLIP) is a major anti-apoptotic protein and an important cytokine and chemotherapy resistance factor that suppresses cytokine- and chemotherapy induced apoptosis. c-FLIP is expressed as long (c-FLIPL), short (c-FLIPS), and c-FLIPR splice variants in human cells. c-FLIP binds to FADD and/or caspase-8 or -10 and TRAIL (TNF-related apoptosis inducing ligand) receptor 5 (DR5- death receptor 5). This interaction in turn prevents (DISC) formation and subsequent activation of the caspase cascade (Safa, 2013).

c-FLIPL and c-FLIPS are also known to have multifunctional roles in various signaling pathways, as well as activating and/ or up-regulating several cytoprotective and pro-survival signaling proteins as NF-κB. In addition to its role in apoptosis, c-FLIP is involved in programmed necrosis and autophagy. Up-regulation of c-FLIP has been found in various tumor types, and its silencing has been shown to restore apoptosis triggered by cytokines and various chemotherapeutic agents. Hence, c-FLIP is an important target for cancer therapy (Safa, 2013).

In the present study, the aim of the work was to evaluate the expression of c-FLIP in MF and its role in the pathogenesis of the disease. The age of the patients ranged from 6 to 60 years with a median age at diagnosis of 38 years. On the contrary, Jawed et al., 2014 reported that MF typically affected older individuals with a median age at diagnosis of 55 to 60 years. They also reported that incidence increased in children and adolescents in the last decades especially in hypopigmented MF. This discrepancy might be related to small number of the studied patients in the present study.

Regarding CD4 expression in MF patients in the current study, there was statistically significant increase in the CD4 density score in patients when compared to controls. There was marked expression of the CD4 lymphocytes in both epidermis and dermis. However, the median level of CD4 expression in the epidermis was higher than the dermis. All patients in this study were classified as patch stage MF except for one case that was plaque stage MF. These findings could explain this heavier epidermal involvement.

In the current study, the c-FLIP intensity score and IIDI were statistically increased in the patients when compared to controls in both epidermis and dermis. These observations were in agreement with a previous study by Stutz et al., (2012) who reported that in both patch and plaque lesions approximately one third of all cases with a phenotype predictive of resistance to apoptosis consisted of Fas-high/c-FLIP-high phenotypes. They also proposed that high expression of the anti-apoptotic protein c-FLIP might play an important role in resistance to apoptosis in those early MF lesions that still exhibit strong Fas expression. The overexpression of c-FLIP acting as an inhibitor of apoptosis is associated with the prolonged chronic course of CTCL.

Also, in vitro studies had clearly shown that c-FLIP expression correlated with resistance to death receptor (DR) induced apoptosis in the skin (Leverkus et al., 2000, Wachter et al., 2004). Furthermore, in other types of malignancy, it was demonstrated that tumors expressing higher levels of c-FLIP were more aggressive and that c-FLIP protected against T-cell mediated cytotoxic immune responses in vivo (Leverkus et al., 2008).

Regarding intensity of c-FLIP immunostaining in different stages of MF, there was an increase in both the c-FLIP intensity score and IIDI with increasing stage of the tumor but it was non statistically significant which may be due to few number of the studied patients in the present study. Leverkus et al., (2008) demonstrated that c-FLIP expression tended to increase with increasing grade of the tumor. This was in accordance with the present study.

Meanwhile, Valente et al., (2006) found that the level of c-FLIP expression didn't correlate with the grade of malignancy. They explained that, at least two possible explanations for such discrepancy could be considered: The first, the control of the extrinsic pathway of apoptosis was strictly dependent on the relative ratio of expression of CD95 and c-FLIP and not on the absolute level of c-FLIP expression. The second, different isoforms of c-FLIP might exert different abilities for controlling chemotherapy- induced apoptosis and the relative ratio of expression of the L- and S-isoforms in low and in high/ intermediate non Hodgkin lymphomas (NHLs) was still unknown. L- and S-c-FLIP isoforms had been shown to act in different ways, and their expression was probably differentially regulated (Micheau et al., 2002).

In the present study, there were statistically significant positive correlations between CD4 and c-FLIP expression in both epidermis and dermis. These observations were in agreement with a previous study by Contassot et al., (2008) who reported that relative expression of c-FLIP had significant positive correlation with CD4 cells.

There was statistically significant positive correlation between c-FLIP expression (in both dermal and epidermal lesions) and the age of patients. This was in agreement with Ili et al., (2013) who reported that patients older than 35 years of age had a greater expression of c-FLIP than younger patients.

Generally speaking, the role of c-FLIP in pathogenesis of MF in this study was confirmed by other studies evaluating its expression in MF and other malignancies. It was demonstrated that defective extrinsic apoptosis is essential for tumor progression of CTCLs and the biological behavior of malignant T-cells could successfully distinguish the clinical heterogeneity of this disease. It

is well recognized that tumor cells are often resistant to chemotherapy via acquiring inhibition of apoptosis (Adedini et al., 2004, Zheng et al., 2014)

Histone deacetylase inhibitors (HDACIs) constitute a group of compounds that promote histone acetylation and transcription of genes involved in multiple cellular processes including apoptosis. Several HDACIs have been proven effective in treating CTCLs. Recent studies showed that apoptosis induced by HDACIs in tumor cells is related to down-regulation of c-FLIP and activation of TNF-related apoptosis-inducing ligand (TRAIL) signaling (Duvic, 2015)

Suberoylanilide hydroxamic acid (SAHA or Vorinostat), an oral HDACIs inhibitor, has proven antitumor activity in a broad range of hematological malignancies and was recently approved by FDA for the treatment of CTCL. Characteristically, the isoforms FLIPL and FLIPS were strongly down-regulated by SAHA in CTCL cells (Al-Yacoup et al., 2012).

Also, proteasome inhibitors such as bortezomib are in clinical development for treatment of myeloma, NHL, and solid tumors. One of their predominant activities is inhibition of NF-kB, which is a main promoter of c FLIP expression. Proteasome inhibition may result in c-FLIP down-regulation and may thereby sensitize CTCL cells for endogenous or exogenous apoptosis (Richardson et al., 2006). Cisplatin (a chemotherapy drug) also was able to inhibit the expression of c-FLIP. It was valuable in treatment of the resistant phenotype of melanoma and ovarian cancer (Song et al., 2003, Abedini et al., 2004).

It therefore seems likely that the evaluation of c-FLIP expression has important role in treatment of CTCL and drugs targeting c-FLIP could help to overcome the resistance to chemotherapeutic drugs, which represents the major issue that frustrates any tentative cure for these malignant tumors.

In conclusion, the expression of c-FLIP may have a role in pathogenesis of MF and a prognostic value as its level increased with tumor progression. Its level also increased with the age of the patients not with duration of the lesions so this might explain the prolonged chronic nature of this disease. Further researches were recommended involving larger number of patients with different stages of the disease, different clinical types of MF, and using different laboratory methods for studying the expression of c-FLIP protein. Also, its effect should be compared with other anti-apoptotic markers to ensure its role in the cancer therapy and measure its expression before and after the treatment.

Statement conflict of Interest

The authors declared that they had no conflict of interest or financial support for this work.

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