BRIEF REPORT

Cytoplasmic increase of Hsp70 protein: potential new biomarker of early infiltration of cutaneous squamous cell carcinoma arising from actinic keratosis

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Abstract:

Background: Cutaneous squamous skin cell carcinoma (SCC) is the second most frequent type of non- melanoma skin cancer and the second cause of death by skin cancer in Caucasian populations. However, at present it is difficult to predict patients with poor SCC prognosis.

Objective: To identify proteins whose expression level could predict SCC infiltration in SCC arising from actinic keratosis (AC).

Methods: A total of 20 biopsies of 20 different patients were studied, 10 were from SCC-AK samples and 10 from normal skin. Early infiltrated SCC-AK was selected on histological examination and to determine the expression of proteins fresh skin samples were processed by 2DE-electrophoresis

Results: The expression levels of three proteins namely alpha-hemoglobin, heat shock protein (Hsp)-27 and 70 were significantly increased in SCC-AK samples with respect to normal control skin. However, only the expression level of Hsp70 protein positively correlated with the level of SCC-AK dermis infiltration. Imnunohistological examination suggested that the increased expression of Hsp70 proteins seems to mainly occur in the keratinocytes cytoplasm. The increased cytoplasmic Hsp70 expression in SCC-AK was confirmed by Western-blot experiments.

Conclusion: Cytoplasmic expression of Hsp70 could be a potential biomarker of early infiltration of SCC arising from an AK.

Keywords: actinic keratosis; cutaneous squamous cell carcinoma; cytoplasm; skin cancer; heat shock protein.

1. Introduction

Actinic keratosis (AKs) is a skin lesion associated with the cumulative effect of the sun. AKs have a risk of progression towards cutaneous squamous cell carcinoma (SCC), also known as epidermoid carcinoma, which is a common form of non-melanoma skin cancer (NMSC) [1,2]. It is estimated that

the risk of progression of an AK to an invasive SCC is between 0.025% and 16% per year, and is the major environmental risk factor for all skin cancer ultraviolet (UV) exposure [3,4].

SCC is the second most common type of nonmelanoma skin cancer. Indeed, SCC caused the majority of NMSC deaths with an incidence of 16 per 100.000 in central Europe, and of 356 per 100.000 sun-exposed white men in USA [5]. Middle-aged and elderly people are relatively more susceptible to this disease than younger people.

Several characteristics have been suggested associated with poor prognostic of SCC, most of them based on structural features of the SCC including tumor thickness, location on the ear, or the desmoplastic histological subtype. In this respect, the Tumor Node Metastasis (TNM) classify SCC on the basis of the SCC diameter, the Broder´s histological degree of differentiation and the invasion of extra-dermal structures [6,7]. However, many other characteristics have been suggested as the worst prognosis such as tumor thickness, location on the ear, or the desmoplastic histological subtype. However, at present it is difficult to predict which patients have higher risk of SCC dermis infiltration, local recurrence or metastases. More over, the importance of tumor thickness as prognostic factor has been also suggested with three main risk categories [7,8]: less than 2 mm with no risk of dissemination, between 2.1 mm and 6.0 mm had a low risk (4%) and greater than 6.0 mm with high risk of metastasis (16%).

However, and taken together it is well recognized that an early diagnosis and outcome prediction probably are the most effective measurements to prevent disease progression and to improve the survival rate of SCC patients.

While the use of biomarkers has become incorporated into the standard of care for numerous malignancies, the application of biomarker studies to predict SCC development has not yet been clearly established. Clinicians can not predict which AK will progress to SCC, which treatment is better for prevention, when some therapies are even contraindicated or when field directed therapies may induce SCC. There has been scarce research about the molecular mechanisms involved in SCC progression, but reported data have suggested that immune-compromised patients, such as organ transplant recipients, presented a higher frequency of SCC and metastasis than the general population [9]. However, there are also several doubts about immunodeficiency since the risk to develop SCC can vary depending upon the immunodeficiency type. Other works have been focused on UV-induced mutations on different genes as the main mechanism to develop SCC from AKs. Differential expression analysis of gene biomarkers has provided very useful information about the molecular behavior of AK and invasive SCC, but the lack of tumor-specific biomarkers remains an ongoing challenge [10,11]. For example, mutation of p53 in SCC has been identified as an early event, and is present in 90% of the SCC but it has also been detected in normal sun-exposed skin [12,13]. Other works have suggested the involvement of proteins involved in the molecular agents involved in cell differentiation, apoptosis, proliferation, migration and cell adhesion.

It could be very interesting to be able to identify biomarkers of early SCC-AK infiltration. New technological approaches to study in a single sample several proteins may be helpful for this proposal. A previous work using long time archival formalin-fixed paraffin embedded samples and proteomics demonstrated differences in the expression of proteins between AK and SCC and particularly in thioredoxin domain-containing 5 protein, a protein-disulphide isomerase that has been reported upexpressed in several other cancers. However, the authors did not analyze changes related to the infiltration degree of SCC and the skin phototype and the histological SCC subtype of the included patients were not specified. Moreover, from a methodological point of view, the efficiency of protein recovery may be modified and influenced by the fixation protocols, fixation time, and sample age [14,15].

Therefore, taken together our aim was to try to identify proteins of early SCC-AK infiltration in fresh skin samples.

2. Results

2.1. Clinical and histological features

The clinical features of the patients showing SCC are shown in Table 1. Six patients were male and four females. Nine of them were phototype II and one phototype I, all with an average solar exposition (Table 1). Five of the SCC were localized on the head (face-ear) and five on the back of the hand.

Six volunteers in the control group were male and four were females. The control volunteers were significantly younger than the SCC patients ($p<0.05$) (Table 1).

All of the included control samples were obtained from a non-photoexposed areas (axila, genitals, scalp and nape). Nine controls were phototype II and one phototype I.

All the SCC showed overlying AK (Table 1), and almost all of them (9/10) also had an AK at the edge (Table 1). Other histological characteristics such as tumor size and infiltration level are also shown in table 1. It is remarkable that dermis infiltration was very initial (Table 1)

Table 1. Characteristics of the patients, controls and skin samples.

VARIABLE		SCC-AK GROUP (n=10)	CONTROL GROUP (n=10)
Gender (M/F)		6/4	6/4
Age		79,80 ±1,41*	47,9 ± 3,36*
Phototype	I	1	1
	II	9	9
Solar exposition	Occasional	----	----
	Average	10	10
	High	----	----
Sample location	Axila-genitals	----	5
	Scalp-nape	-----	5
	Face-ear	5	-----
	Back hand	5	-------
AK overlying the SCC		10/10 (100%)	
AK at the edge of the SCC		9/10 (90%)	
Size (cm)		1,85 ± 0,11	
Dermis Infiltration (mm)		1,57 ± 0,35	
Level of infiltration (cm)		2,9 ± 0,94	
Foci of ulceration		1/10 (10%)	
Adnexal involvement		0/10 (0%)	
Elastosis in dermis		10/10(100%)	

Results are represented as mean ± SD. Abbreviations: M: male; F: female; SCC-AK: squamous cell carcinoma arising in AK; cm: centimeters; mm: millimeters; AK: actinic keratosis; SCC: Squamous cell carcinoma; *$p<0.05$ with respect to SCC.

2.3. Two-dimensional electrophoresis analysis

Two-dimensional (2-DE) electrophoresis spots were analyzed and identified on the basis of a previous published work showing the human skin map proteome [16]. The measured spots were chosen when spots were at least expressed in 65% of the 2-DE gels within each of the two groups of skin samples, control and SCC samples. Proteins were densitometrical analyzed and classified with respect to their main functional characteristic: structural proteins, heat shock proteins, antioxidant proteins, tumor markers proteins, transport protein and transcription factor protein.

There were no statistical differences between control and SCC samples in the level of expression of structural proteins namely actin, annexin I, two identified isoforms of annexin IV, two isoforms of annexin V and two isoforms of cytokeratin (Table 2). There were also no differences in the level of expression of proteins associated with antioxidant, transcriptional mechanisms (Table 2).

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The expression level of the alpha-hemoglobin was significantly higher in SCC samples with respect to control (Table 2). The expression level of the Hsp-27 and Hsp-70 proteins was also higher in SCC samples than in control (Table 2).

Table 2. Comparison of the protein expression profile in normal skin (controls) and SCC-AK.

PROTEIN	CONTROLS (N=10)	SCC-AK (N=10)	P VALUE
Structural proteins			
Actin	130,29 ± 49,14	271,60 ± 80,55	0,102
Anexin I	75,40 ± 37,00	44,72 ± 16,37	0,876
Anexin IV			
Isoform 1	47,59 ± 14,51	53,20 ± 18,27	0,684
Isoform 2	32,68 ± 19,96	15,63 ± 8,99	0,400
Anexin V			
Isoform 1	39,95 ± 12,23	36,15± 8,51	0,905
Isoform 2	35,65 ± 14,63	35,39 ± 8,96	1,000
Cytokeratin			
Isoform 1	29,26 ± 9,77	34,10 ± 7,58	0,497
Isoform 2	26,99 ± 11,04	13,47 ± 2,89	0,431
Calreticulin			
Isoform 1	28,21 ± 7,44	23,16 ± 7,15	0,497
Isoform 2	28,79 ± 13,78	25,84 ± 10,51	1,000
Isoform 3	20,29 ± 10,00	19,56 ± 11,72	0,898
Heat shock proteins			
Hsp70	17,28 ± 4,11	42,20 ± 8,34	**0,035**
Hsp27	78,11 ± 49,03	308,50 ± 125,79	**0,006**
Antioxidant protein			
Glutathione-S-Transferase	24,67 ± 9,98	29,92 ± 6,48	0,195
Tumor markers			
Maspin	30,39 ± 12,15	22,86 ± 10,48	0,370
SCCA-2	83,78± 29,81	174,04 ± 50,00	0,423
Transport proteins			
Alpha-hemoglobin	32,63 ± 9,94	98,68 ± 18,11	**0,006**
Apo-AI	72,57 ± 23,43	74,79 ± 19,79	0,796
Transcription factor			
Rho-GDP	46,63 ± 19,63	27,53 ± 9,76	0,549

Results presented as mean ± SD. Abbreviations: SCC-AK: Squamous Cell Carcinoma over an AK; Hsp: Heat-shock protein; SCCA-2: Squamous Cell Carcinoma Antigen 2; Apo-AI: Apolipoprotein A1; Rho-GDP: Rho-Guanosine Triphosphate.

2.4. Correlations between the protein expression and the histopathological characteristics of the SCC-AK.

The relationship between the infiltration degree of SCC-AK and the level of expression of the proteins that reached statistical differences between control and SCC-AK samples were analyzed (Table 3). Considering all the infiltration degrees, there was no association between it and the level of expression of either alpha-hemoglobin Hsp27 and Hsp70 proteins (Table 3). However, Spearman analysis revealed a positive correlation between the level of expression of Hsp70 and infiltration SCC-AK levels II and III (Table 3). The statistical significance was lacking when a more advanced infiltration degree was correlated with the expression level of either alpha-hemoglobin and Hsp27 and 70 (Table 3). Therefore, only early phases of dermis infiltration of squamous epithelial cells were associated with the expression level of Hsp70 (Table 3).

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Table 3. Spearman associations between level of SCC infiltration and the protein expression level of Hsp27, Hsp70 and alpha-hemoglobin.

PROTEINS	CORRELATION (Spearman, rho)	P VALUE	IHQ Hsp70 Nucleous	IHQ Hsp70 Citoplams
All SCC-AK levels of infiltration (n=10)				
Alpha-hemoglobin	0,273	0,446		
Hsp27	-0,176	0,627	Mild + (10/10)	Moderate++ (7/3) High+++(3/3)
Hsp70	-0,212	0,556		
Levels of infiltration II y III (n=6)				
Alpha-hemoglobin	0,200	0,704		
Hsp27	0,429	0,397	Mild+ (6/6)	Moderate++(3/3) High+++ (3/3)
Hsp70	**0,829**	**0,042**		
Level of infiltration IV (n=4)				
Alpha-hemoglobin	0,400	0,600		
Hsp27	- 0,400	0,600	Mild+ (4/4)	Moderate+++ (4/4)
Hsp70	-0,600	0,400		

*Classification in Clark levels; SCC: Squamous cell carcinoma over an AK;

2.5. Hsp70 expression in SCC-AK

Immunohistochemistry analysis demonstrated that healthy epidermal keratinocytes expressed Hsp70 protein at cell nucleus (Figure 1). A slight hsp70 expression was also observed in the cytoplasm of control keratinocytes (Figure 1). In SCC-AK samples with slight SCC infiltration (level II and III), Hsp70 staining was greater than in control. Indeed, in these SCC-AK samples, Hsp70 protein was also localized in both the cytoplasm and in the nucleus but particularly in the cytoplasm the expression level of HSP70 protein was markedly greater than in that cytoplasm from control samples (Figure 1). This overexpression of Hsp70 protein was found in all the six SCC-samples showing slight SCC infiltration (+) and they were classified as intense in 50% of the cases (+++).

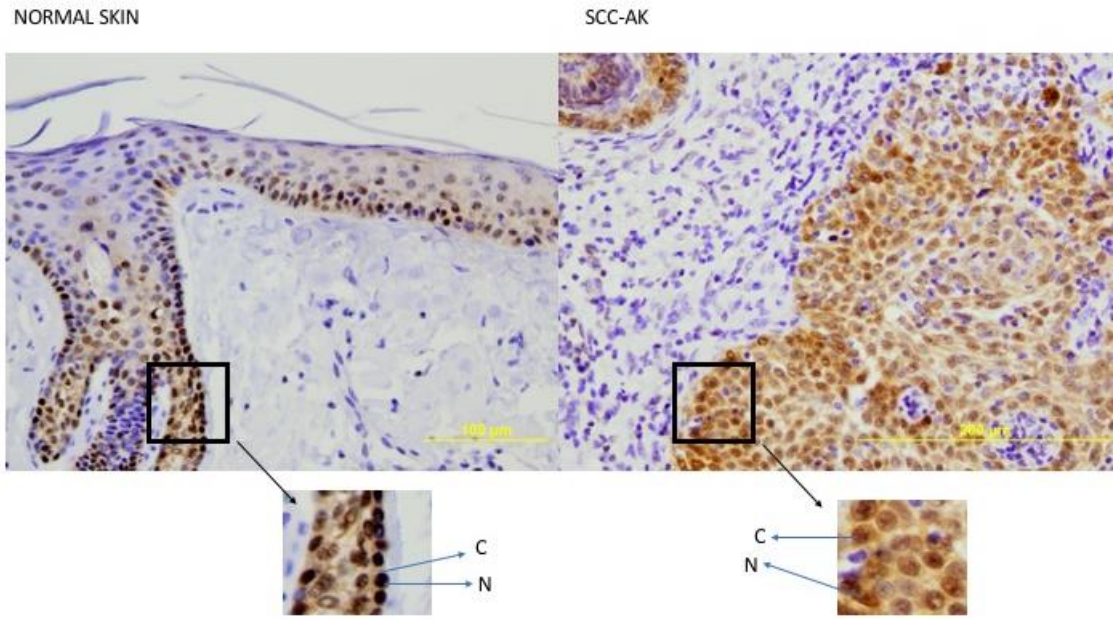

Figure 1. Immunohistochemistry analysis showing Hsp70 expression in control (normal skin 40x) and SCC-AK skin samples with a microinfiltrating focus of SCC (40x). Abbreviations: squamous cell carcinoma over actinic keratosis (SCC-AK), nucleus (N) and cytoplasm (C).

The expression level of cytoplasmic Hsp70 protein in SCC-AK samples was also analyzed by Western blotting. As figure 2 shows, higher significant cytoplasmic expression of Hsp70 protein was observed in SCC-AK samples as compared with control (Figure 2). Cytoplasmic Hsp70 upexpression remains higher in SCC-AK than in control when age was used as covariant.

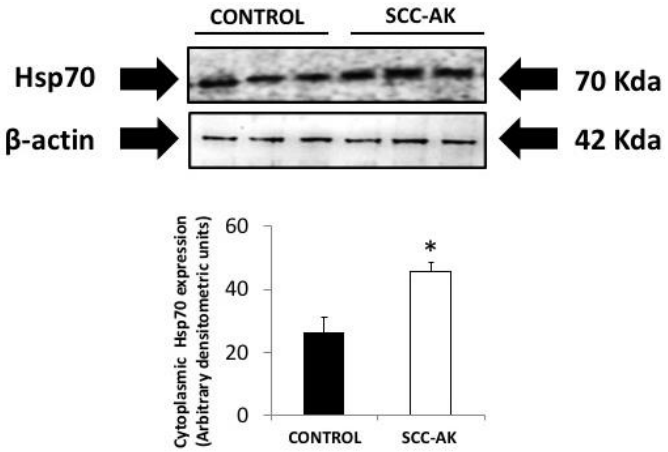

Figure 2. Representative Western blot experiments showing the expression of Hsp70 protein in cytoplasm from control and SCC-AK samples. β-actin was used as loading control. Bar graphs show the cytoplasmic expression Hsp70 levels of all the Western blots. Results are represented as mean±SD. *p<0.05 with respect to control.

3. Discussion

The present work shows, in our knowledge for the first time, an association between Hsp70 expression and the early infiltration of SCC-AK.

A biomarker that can stratify the risk of transformation of AK into SCC has still not been identified. Furthermore, the field directed therapies used to reduce this risk [17], have demonstrated inducement of carcinogenesis in the field treated [18,19,20]. Therefore, dermatologists need to be able to select the treatment and the risk-patients towards a personal medical treatment.

The cautious selection of the tumors and the patients studied is critical, as AK and SCC shared biomarkers between them and with normal photoexposed skin [12,13]. Immunosuppression, chronic solar exposition and fair skin are known risk factors for SCC [3,9,10]. All of the included patients with SCC had similar phototypes and patterns of solar exposition and none of them were immunosuppressed patients [21,22].

The SCC included in this study were low risk SCC according to their differentiation degree, size (< 2cm), depth of infiltration (<2 mm) and histological features [6-8, 23]. All of them appeared over an AK, being representative of the most frequent type of SCC in clinical practice, low-risk SCC-AK and the first step of an AK developing a SCC [4, 24-27]. These criterium were considered important to identify possible early markers of SCC-AK infiltration.

Patients in the control group were comparable in terms of photoype, solar exposition and gender, but were younger than the SCC group (p>0.005). The control normal skin was located in none photoexposed areas (axilla, genitalia) to minimize molecular and histological changes caused by ultraviolet radiation [12,13].

The molecular data suggested differences between control skin and ASC-AK samples in the expression level of three proteins namely alpha hemoglobin and two Hsp proteins, Hsp27 and Hsp70. However, Spearman analysis suggested that only the expression of Hsp70 protein was associated with SCC infiltration. Interestingly such association was only significant at low infiltration levels (levels II and III) but not at higher infiltration degree (level IV). It suggests the specificity of Hsp70 up-expression to identify low SCC-AK infiltration.

Hsp27 and hsp70 showed higher expression in SCC than in normal skin. Hsp 27 and 70 have a protective role in stress and develop a variety of functions, including regulating the cell growth and differentiation. Interestingly, both proteins have been reported to play a role in chemotherapy

resistance [43]. Indeed, Hsp27 was reported abundantly expressed in malignant cells and has been described as a prognostic factor and metastatic potential marker in gastric, colon and esophageal cancer [44]. On the other hand, overexpression of Hsp70 has been associated with invasion and poor prognostic in patients with OSCC and consequently as a promising target for treatment [45]. Alpha-hemoglobin has also shown significative increased expression in SCC-AK. Expression of globin chains has been reported in a number of non-erythroid tissues, including neuronal cells, mesangial cells, macrophages and hepatocytes, as well as in several types of epithelial cancers.[28]. The functional significance of globin chain expression in the cells and tissues remains to be elucidated, but has been suggested to provide oxygen storage in the tissue cells. Interestingly, Zheng et al have suggested that beta-globin enhancing anchorage-independent lung and breast cancer cells survival facilitate distant metastasis [29].

Interestingly, immunohistochemistry experiments supported the existence of higher expression of SCC-AK and it was specially increased in the cytoplasm. In this regard, Western blot experiments also demonstrated significant higher expression of Hsp70 protein in cytoplasm from SCC-AK cells than in control keratinocytes. This higher cytoplasmic Hsp70 expression in SCC-AK cells with respect to control was independent of age as was suggested by the fact that in the regression analysis cytoplasmic Hsp70 levels remained significatively increased in SCC-AK with respect to control after using age as covariant..

Hsp70 has been involved in increased tumor growth and metastatic potential by inhibiting cell apoptosis. In other cell types, depletion or inhibition of Hsp70 frequently reduces the size of the tumors and can even cause their complete involution. In this respect, Hsp70 has the ability to interact with different cytoplasmatic proteins involved in the cellular apoptosis regulation. Indeed, it has been described that cytosolic Hsp70 interacts with BAG-1, an apoptotic blocker protein, through its binding to the anti-apoptotic protein Bcl-2 [30] Moreover, it was described that Hsp70 promotes mitogen-activated protein kinases (MAPs) phosphorylation inducing growth and cellular survival and block the pro-oncogen BAX into mitochondria, avoiding cellular apoptosis [31].

Previous works have demonstrated Hsp70 upexpression in non-skin SCC. Xiaoping Wang found significant correlation between HSP72 (a subgroup of HSP70) and progression of esophageal SCC [32]. However, a study in paraffin-embedded biopsy specimens of SCC from various sites of oral and para-oral regions did not find changes in Hsp70 expression [33]. These different results may be related to specific characteristics of SCC that may include from the sample type to the SCC infiltration degree that may promote distinct molecular behaviors.

Study limitations

The main limitation of the present study may probably be the reduced sample size. However, the results were very consistent, and their specificity is also supported by the fact that by Spearman associations only a significative correlation with one of the all studied proteins was found. In addition, the Hsp70 expression level was only associated with a specific SCC infiltration degree. However, we are aware that additional studies with greater patient´s number are needed.

Another study limitation may be the different age between control and AK-SCC patients. It is probably because, control skin was extracted from the leftover skin of benign excisions, which are more frequent in younger patients. However, as previously mentioned, changes in cytoplasmic Hsp70 expression observed in AK-SCC with respect to control were not age dependent since the significative differences remained after using age as covariant in the linear regression model.

4. Materials and Methods

4.1. Collection of skin samples

Cutaneous samples (size <5 mm) of well-differentiated cutaneous squamous cell carcinoma from sun-exposed regions (head, neck, dorsal hands) were obtained from ten patients during micrographic surgery. Ten normal specimens were also obtained from <5-mm punch biopsies from non-sun-exposed areas of ten normal volunteers. The demographic data of all patients (age, gender and

phototype), the sample location and the punctuation in the questionnaire of solar exposition validated for the Spanish population were collected [22]. None immunosuppressed patients with similar phototypes and patterns of solar exposition were included. Patients taking thiazide diuretics were excluded. In addition, patients with systemic inflammatory, infectious or other oncological diseases, and/or subjected to any surgical procedure within the last 6 months were excluded. The work was performed according to principles outlined in the Declaration of Helsinki. All the included individuals signed the inform consent and the local institutional Ethics Committee approved the study (Internal register number: FCS/2015/3)

4.2. Histological examination

A routine hematoxilyn-eosin histological study was performed in the biopsies. An expert dermatopatholigist studied the tumors and only SCC arising over an AK were selected. In the SCC studied, the presence of an AK at the edge, the size in centimeters, the infiltration in dermis in micra, the level of infiltration (Clark, 1-5), the presence of foci of ulceration, the adnexal involvement and the elastosis in dermis was described.

4.3. Two-dimensional electrophoresis (2-DE) of plasma proteins, image acquisition and analysis.

As previously reported in detailed, 2-DE 250 was performed loading 250g of total protein was used [34,35]

Samples were loaded on immobilized gradient IPG strips (18 cm, pH 4-7) and isoelectric focusing was performed using a Protean IEF cell system (Bio-Rad). In the second dimension, proteins were resolved on 10% SDS-PAGE gels using a Protean II XL System (Bio-Rad). As previously reported, the gels were then fixed, silver stained during 30 min and scanned in a UMAX POWERLOOK III Scanner. One 2-DE gel was performed for each sample and all of the here-identified spots were at least expressed in 70% of the 2-DE gels. The spots were densitometric analysed by using the Quantity One 4.2.3. software (Bio-Rad). The densitometric intensity of each spot was evaluated after subtracting background staining of the corresponding gel.

4.4. Immunohistochemical examination of Hsp70 in SCC

Histological preparations of 5 microns thickness from different regions of the SCC and controls were assessed to study the presence of Hsp70. An internal and external control of the procedure was used (normal skin). Immunohistochemical technique was performed using the Hsp70 monoclonal antibody (Ab2787) tested for in vitro studies in humans following the manufacturer's instructions (Diagnostic Master®, Vitro). Two dermatopathologists assessed the subcellular localization of Hsp70 and quantified the intensity in mild (+), moderate (++) and high (+++) in all samples. The correlation between observers was also assessed.

4.5. Cytoplasmic Hsp70 expression in cytoplasmi by Western blot

The cytoplasmic expression level of the Hsp70 protein was analyzed by Western blot. For this purpose, biopsies were lysed and cytoplasmic fraction isolated following the instructions of a commercial kit (Mitochondrial isolation kit, Thermofisher, CA) The cytoplasmic fraction was solubilized in Laemmli buffer containing 2-mercaptoethanol. The proteins were separated in denaturing gels of polyacrylamide and 15% SDS. The same amount of protein (40 g/well) was loaded into each well. To detect the protein expression level, proteins in gels were transferred to nitrocellulose membranes (Immobiliun-P, MiliporeÒ) and blocked for 1 hour at room temperature in a buffer containing 5% BSA. After blocking, membranes were incubated with monoclonal antibody against the Hsp70 protein (1: 1000; ab2787, AbcamÒ) and then incubated with secondary antibody (horseradish peroxidaxe conjugated IgG anti-rabbit) at 1: 2500 dilution. Proteins were detected by enhanced chemiluminescence (ECLÒ, Amersham Biosciences) and evaluated by densitometry (Quantity OneÒ, Bio-Rad Laboratories). Molecular weight markers (Sigma, St Louis, MI, USA) were used for molecular mass determination. In order to compare the protein expression of the different

proteins with the expression of another constitutive protein, the expression of β-actin was also analyzed. For this, a parallel gel was run with the same samples and then the membrane transfer was incubated with a monoclonal anti-β-actin antibody (1: 2000, Sigma-Aldrich, Saint Louis, USA) and respective secondary antibody (IgE anti-Rabbit) at a 1: 2500 dilution.

4.6. Statistical analysis

Results are expressed as mean ± S.E.M. To compare the levels of protein expression between the control groups, the non-parametric Mann-Whitney test was used. Correlation analysis between tumor depth and the expression of proteins were performed by Rho-Spearmen test. To control the influence of age on Hsp70 expression a linear regression analysis was performed. The dependent variable was the level of Hsp70 expression, the independent variable was the experimental groups (Control, SCC-AK) and age was used as covariant. The SPSS version 22.0 program (SPSS Inc.) was used. A value of $p < 0.05$ was considered statistically significant.

5. Conclusions

Slight dermis infiltration (level II and III) of SSC-AK was associated with higher Hsp70 levels in the biopsies. Hsp70 levels seems to be mainly increased in the cellular cytoplasm. This finding suggests Hsp70 as a potential early biomarker of SCC-AK infiltration and therefore, further studies are needed to confirm this finding

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