

CD1A-positive cells and HSP60 (HSPD1) levels in keratoacanthoma and squamous cell carcinoma

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Abstract CD1a is involved in presentation to the immune system of lipid antigen derived from tumor cells with subsequent T cell activation. Hsp60 is a molecular chaperone implicated in carcinogenesis by, for instance, modulating the immune reaction against the tumor. We have previously postulated a synergism between CD1a and Hsp60 as a key factor in the activation of an effective antitumor immune response in squamous epithelia. Keratoacanthomas (KAs) are benign tumors that however can transform into squamous cell carcinomas (SCCs), but the reasons for this malignization are unknown. In a previous study, we found that CD1a-positive cells are significantly more numerous in KA than in SCC. In this study, we analyzed a series of KAs and SCCs by immunohistochemistry for CD1a and Hsp60. Our results show that the levels of both are significantly lower in KA than in SCC and support the hypothesis that KA may evolve towards SCC if there is a failure of the local modulation of the antitumor immune response. The data also show that immunohistochemistry for CD1a and Hsp60 can be of help

in differential diagnosis between KAs and well-differentiated forms of SCC.

Keywords Keratoacanthoma · Squamous cell carcinoma · Hsp60 · CD1a · Immunohistochemistry · Differential diagnosis · Prognostic evaluation · Treatment

Introduction

KA is a relatively common crater-like squamo-proliferative lesion clinically characterized by a rapid onset and usually by spontaneous slow regression within months, if left untreated (Blessing et al. 1994). Sometimes, KA may be difficult to distinguish from a well-differentiated SCC with a crateriform architecture both clinically and histologically. In fact, some forms of KA are defined “atypical” due to the presence of focal areas with some features overlapping those of SCC, consisting of a higher degree of cytological atypia, a higher nuclear/cytoplasmic ratio, several—sometimes atypical—mitosis, and focal aspects of irregular infiltration at the deep boundary level. SCC appears as a persistent, slow-growing, non-healing lesion, with an inexorable progression characterized by local growth, tissue destruction, and even metastasis. Despite the fact that KA and SCC have been considered to be two separate entities (Mukunyadzi et al. 2002; Schwartz 2004), the presence of overlapping features and the possibility of malignant transformation, especially in older patients and in photoexposed areas (Sanchez et al. 2000), has recently led to the hypothesis that the two neoplasias might well represent the two extreme ends of the same spectrum, with forms of transition and possibility of evolution of KA toward SCC (Fernandez-Flores 2005; Cabibi et al. 2011).

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Two fundamental issues in carcinogenesis are the mechanism by which a normal cell becomes malignant and the biomarkers that predict malignant progression.

Hsp60 (or HSPD1, Kampinga et al. 2009) is a molecular chaperone localized mainly in mitochondria and, to a lesser extent, in the cytosol (Itoh et al. 2002). Its classical role pertains to protein folding within mitochondria in association with Hsp10 (Martin 1997). Hsp60 also controls cell proliferation and tumorigenesis by interacting with mitochondrial Hsp70 (i.e., mortalin) and other tumor-related molecules (Dundas et al. 2004; Wadhwa et al. 2005; Deocaris et al. 2006). A progressive increase in Hsp60 levels is associated with progression from dysplasia to carcinoma in the carcinogenesis process of the uterine exocervix (Cappello et al. 2002), large bowel (Cappello et al. 2003a, 2005a), and prostate (Cappello et al. 2003b). When increased in tumor cells, Hsp60 accumulates in the cytosol and is secreted via the exosome and/or the Golgi pathways (Merendino et al. 2010; Campanella et al. 2012). In contrast, Hsp60 levels progressively decrease during bronchial (Cappello et al. 2005b, 2006a) and oral carcinogenesis (Ito et al. 1998) as well as during bladder cancer progression (Lebret et al. 2003; Cappello et al. 2006b), although the latter has not been confirmed by others (Romanucci et al. 2012). For these reasons, Hsp60 has been dubbed as the “molecular proteus” of carcinogenesis (Cappello and Zummo 2005): It has a dual behavior changing it most likely as dictated by the tumor microenvironment.

CD1a cells belong to the CD1 family and are involved in self and foreign lipid antigen presentation to T cells (Vincent et al. 2005). CD1a cells present lipid antigen derived from apoptotic or necrotic cells occurring in the tumor microenvironment (Coventry and Heinzl 2004). The presence of CD1a+ dendritic cells (DCs) infiltrating the tumor correlates with a better prognosis in mammary (Hillebrand et al. 1999; Bell et al. 1999; Treilleux et al. 2004; La Rocca et al. 2008), oesophageal (Ikeguchi et al. 1998), and colorectal (Sandel et al. 2005) cancers. CD1a+ tumor-infiltrating DCs can recognize and process tumor antigens but to achieve full functional efficiency, they migrate, after antigen endocytosis and processing, into lymph nodes, in which their full maturation takes place (Adam et al. 2003; Andrews et al. 2005). Previously, we found that intratumoral and peritumoral CD1a cells are significantly more numerous in KA than in SCC (Cabibi et al. 2011). Moreover, in the past, we postulated a synergism between CD1a cells and Hsp60, which can activate cells of the innate immune system, causing the release of Th1 cytokines, as well act as a danger signal and promote maturation of DCs (Corrao et al. 2008; Chen et al. 1999; Flohé et al. 2003).

Consequently, we asked the questions: What are the quantitative levels of Hsp60 in relation to those of CD1a cells in KA? Can these levels help in identifying cases that progress to the malignant SCC? Our prediction was that low Hsp60 levels

accompanied by low levels of CD1a cells would indicate malignant progression. The work reported here pertains to these issues.

Materials and methods

We examined retrospectively 18 cases of KA and 12 cases of SCC. All the cases were submitted to the Department of Human Pathology of the University of Palermo from January 1, 2012 to September 2, 2014. The age of the KA patients ranged from 20 to 80 years (mean 62 years), the mean tumor diameter was 0.93 cm (range 0.5–1.3 cm), and the time from onset was on average 3 months (range 1–4 months). The SCC patient's age ranged from 50 to 90 years (mean 74 years), the mean tumor diameter was 1.58 cm (range 0.3–4.5 cm), and the time from onset was on average 31 months (range 1.5–38 months). The stored slides, stained with haematoxylin-eosin, were re-evaluated by two pathologists (DC and FR). The SCC cases were subdivided into two groups. One group consisted of 8 cases of well differentiated or with an intermediate grade of differentiation of SCC (SCC G1-G2); the other group consisted of 4 cases of SCC with a low grade of differentiation, i.e., greater malignancy (SCC G3).

This study was approved by the local ethics committee, and informed consent was obtained from the patients before the surgery. Immunohistochemistry was performed as described (Cabibi et al. 2011) using the following primary antibodies: anti-CD1a (monoclonal mouse antibody, clone 010, DAKO, Carpinteria, CA, USA; dilution, 1:50) and anti-Hsp60 (monoclonal antibody, clone LK1, Sigma-Aldrich Inc., Milan, Italy; dilution, 1:400). Immunohistochemical results were evaluated by two experts (DC and FC) in a blind way, with coded slides. Tissue positivity for Hsp60 was evaluated with a semi-quantitative method as follows: score 3+ => >70 % of positive cells, score 2+ = 30–70 % of positive cells, score 1+ = 10–29 % of positive cells, score 0 = <10 % of positive cells. CD1a cells were evaluated quantitatively as percentage of positive intra- and peri-tumoral cells. Ten microscopic fields at $\times 200$ magnification for each slide were examined, and the arithmetic means were considered for statistical analyses. Statistical analyses were performed by using the software GraphPad Prisma 5. The positive cell distribution was compared in the 3 groups (KA, SCC G1-G2, and SCC G3) by using the Mann-Whitney *U* test. A *p* value ≤ 0.05 was considered significant.

Results

Both Hsp60 and CD1a cells were more abundant in KA than in SCC. All KAs showed a score 3+ for Hsp60, and the mean percentage of CD1a cells was 27 % (SD: 5.5). In SCC, Hsp60 levels were lower: all SCCs G1-G2 showed score 2+, while

SCC G3 showed score 1+ (2 cases) and score 0 (2 cases). Noteworthy, in SCC G2, Hsp60 showed a heterogeneous quantitative distribution pattern, with positivity maintained in some cells and reduced or absent in others, which generated a mosaic design made of cells differing in Hsp60 positivity but otherwise similar, i.e., not showing any other morphological differences (Figs. 1 and 2). In SCCs, CD1a cell levels were reduced too (mean value=9.14 % SD 1.75) with statistically significant difference between SCC and KA ($p<0.05$). In the SCC patients, no significant differences were evident between G1–G2 group and G3 group: G1–G2 cases showed 8.5 % CD1a cells (SD: 1.25); G3 cases showed 9.75 % CD1a cells (SD: 2.25) (Fig. 1). The results are graphically represented in Fig. 3. Remarkably, when present within well-differentiated subregions of a tumor, e.g., in SCC G3, the cells were significantly more positive for Hsp60 than those in the less differentiated subregions of the same tumor. Similarly, in poorly differentiated areas (G3 areas), occurring within a well-differentiated SCC, the cells had lower levels of Hsp60 than in the rest of the tumor (Fig. 4).

Discussion

Our data point to a simultaneous decrease in the levels of Hsp60 and CD1a cells that would occur only in KAs that progress to SCC, which makes this quantitative pattern a

marker of malignant progression. Tissue assessment of Hsp60 and CD1a is easy to perform and could facilitate the task of the pathologist as a useful adjunct in the diagnosis of difficult, atypical cases of KA.

Investigations on the role of Hsp60-CD1a cells interplay in carcinogenesis pertaining to KA ought to consider what is already known on this very topic and what is still obscure. For instance, the mechanisms initiating KA and its progression to SCC are still unclear. Epidemiological studies have indicated that risk factors for KA include sun exposure, chemical carcinogens, viruses, cutaneous infection, and trauma. KA has been reported to occur in sites of previous cryotherapy, topical photodynamic and UV therapies, megavoltage radiotherapy, split skin graft donor, excisional surgery scars, and tattoos (Swaw et al. 1990; Hendricks and Sudden 1991; Pattee and Silvis 2003; Craddock et al. 2004; Kimyai-Asadi et al. 2004; Maydan et al. 2006; Brazzelli et al. 2006; Kaptanoglu and Kutluay 2006; Kluger et al. 2008). Many of these procedures are considered to cause local stress that induces Hsp60 overexpression (Jalili et al. 2004; Singh et al. 2009; Henderson 2010). Hsp60 accumulates not only in tumor cells but often also in pre-tumor dysplastic lesions, such as the squamous intraepithelial lesion of exocervix (Cappello et al. 2002). When accumulated inside a cell, Hsp60 can be actively released by the cell into the extracellular space and circulation and, thus, elicit an immune response (Chen et al. 1999; Flohé et al. 2003). It has been suggested that Hsp60 can activate cells

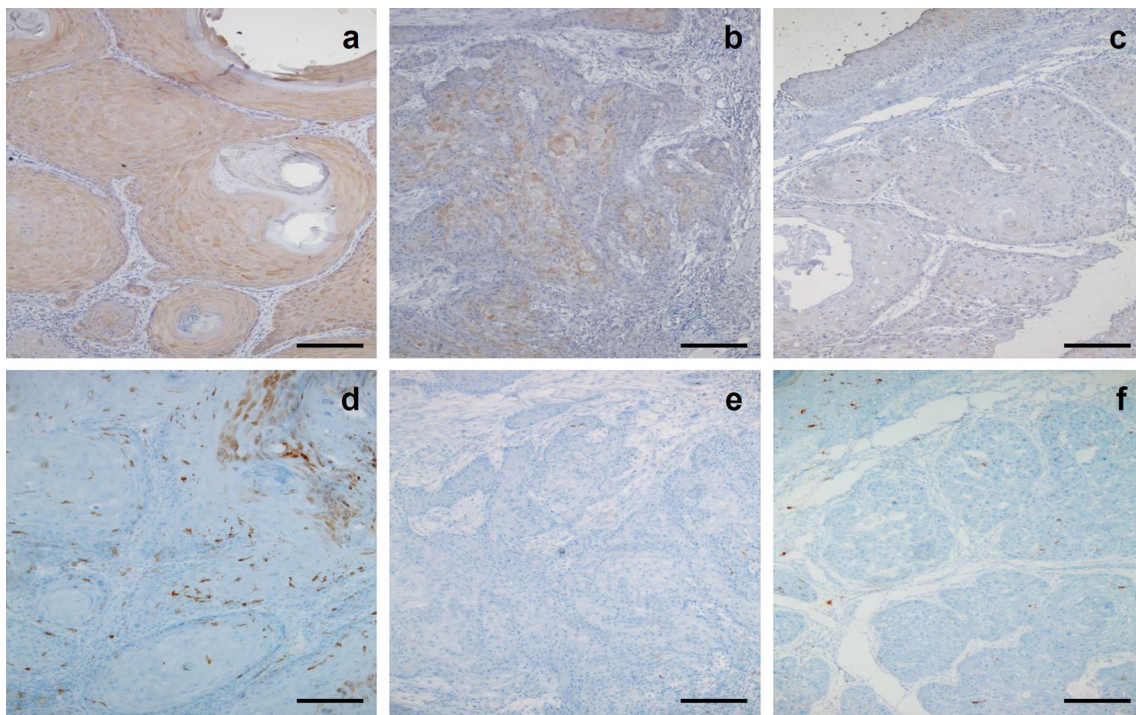


Fig. 1 Immunohistochemical staining for Hsp60 in KA (a), SCC G1-G2 (b), and SCC G3 (c); and CD1a in KA (d), SCC G1-G2 (e), and SCC G3 (f). High levels of Hsp60 and CD1a in KA; progressive decrease of

Hsp60 and CD1a-positive immunostaining in G1-G2 and G3 SCC, with a mosaic pattern in G2 cases. Magnification $\times 200$. Bar: 100 μm

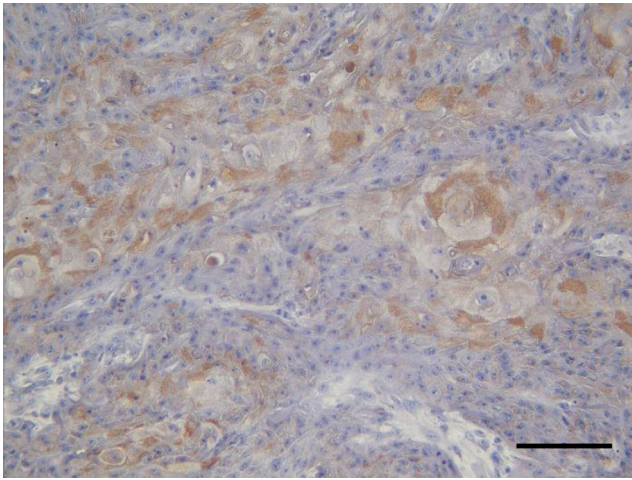
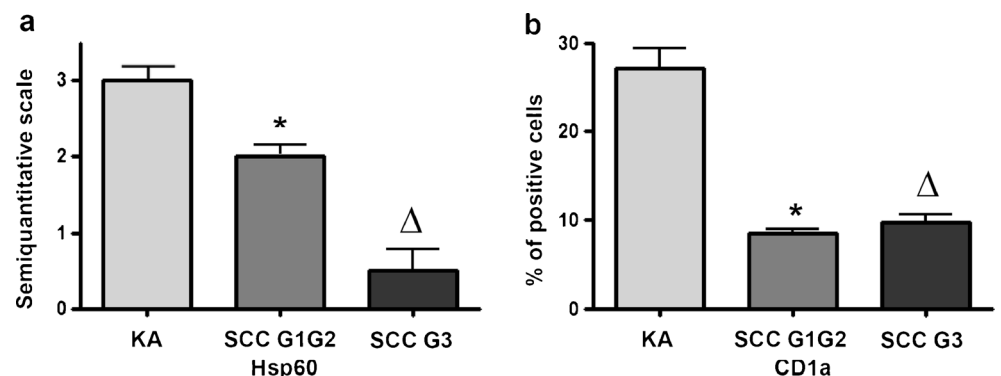


Fig. 2 Heterogeneous diminution of Hsp60 levels in SCC G2, with positivity maintained in some cells and decreased or absent in others, creating a mosaic pattern. Magnification $\times 200$. Bar: 100 μm

of the innate immune system, determining the release of Th1 cytokines (Chen et al. 1999), as well as act as a danger signal and promote maturation of DCs (Chen et al. 1999; Flohé et al. 2003). Hsp60 can also stimulate B cells via TLR4-MyD88 signaling and, thereby, promote their proliferation, the expression of costimulatory molecules, and the secretion of Th2 cytokines (Cohen-Sfady et al. 2005). It has been shown that Hsp60 is also able to interact with TLR2 on T cells, in turn inhibiting the cytoskeletal rearrangement and the chemotaxis induced by chemokine stromal cell-derived factor-1 α (Zanin-Zhorov et al. 2003, 2005). Hsp60 also colocalizes with both CD14 receptor and lipopolysaccharide-binding sites, stimulating the immune response. Hsp60 acts together with a receptor complex (consisting of CD14 co-receptor and TLR4 signaling receptor) enhancing IL-12 production by antigen-presenting cells (APCs) and the IFN γ release by T cells, whose activation strictly depends on the presence of professional APCs, such as DCs (Osterloh et al. 2007). Furthermore, Hsp60 can activate the innate immune response also by chaperoning pro-inflammatory mediators (Tsan and Gao 2004).

Fig. 3 The histograms show the immunopositivity for Hsp60 (a) and CD1a (b) in KA, SCC G1-G2, and SCC G3. Statistical analyses demonstrated significant differences between KA and SCC G1-G2 and between SCC G1-G2 and SCC G3. * and Δ mean $p < 0.05$



* $\Delta p < 0.05$

CD1a antigen is constitutively expressed in DCs, Langerhans' cells, and thymocytes (Coventry and Heinzel 2004) in which it is present on the cell membrane associated with $\beta 2$ -microglobulin (Porcelli et al. 1998). CD1a+ Langerhans' cell precursors are recruited within sites of injury in response to secreted chemokines (Dieu-Nosjean et al. 2000). In this way, CD1a antigen may contribute in the activation of the immune system that, in turn, may generate an antitumor response (Coventry and Heinzel 2004).

We have postulated a synergistic action of Hsp60 and CD1a cells in pre-tumoral and tumoral squamous epithelia (Corrao et al. 2008). Particularly, because of the CD1a antigen and Hsp60 involvement in activating the innate immune system response, we hypothesized that when these two molecules are co-expressed in a pretumoral tissue, their antitumor immune effects would be amplified (Corrao et al. 2008). Indeed, Hsp60-positive cells that have undergone a dangerous stress can release Hsp60 that can act as chaperone for lipid antigens to CD14/TRL4 receptors expressed on immature DCs. During pre-tumoral lipid-antigen processing, DCs migrate and mature into the lymph nodes and, finally, become able to present pre-tumoral lipid-antigen to the CD1a positive cells, in turn activating the antitumor immune response (Coventry and Heinzel 2004).

In this work, we found that levels of Hsp60 and CD1a cells are low in SCC as compared to KA and one may argue that when their synergistic action falls below a certain level, KA can progress to SCC. One may postulate that low levels of Hsp60, such as those found during cancer progression in other squamous stratified epithelia like those of the oral, bronchial, and urothelial mucosa (Cappello et al. 2005b, 2006a, b; Ito et al. 1998; Lebret et al. 2003), are responsible for the reduction of the number and, consequently, of the anti-tumoral activity of CD1a cells in KA.

Typically, CD1a cells present lipid antigen derived from apoptotic or necrotic cells to T cells (Coventry and Heinzel 2004). In a previous work, we measured the lymphocytic infiltration in KA and SCC by using anti-CD3, -CD4, -CD8, -CD20, and -CD57 antibodies (Cabibi et al. 2011). We did

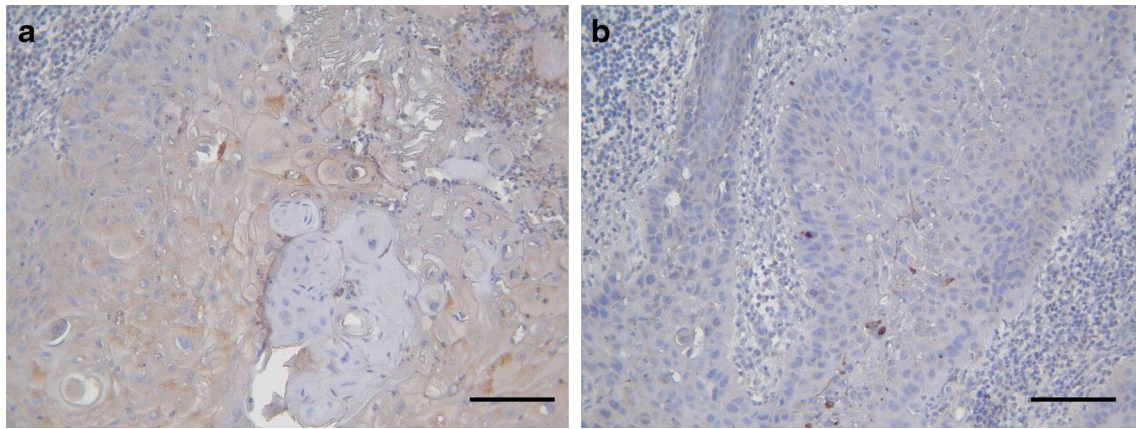


Fig. 4 Representative images of immunohistochemistry for Hsp60 in SCC G3. The well-differentiated areas within the tumor tissue were more positive for Hsp60 (a) as compared with the less differentiated areas of the same specimen (b). Magnification $\times 200$. Bar: 100 μm

not find significant differences between the groups for CD3, CD4, CD8, and CD20. However, the mean number of peritumoral CD57+ cells in KA was significantly higher than in SCC while the mean number of intratumoral CD57+ cells was extremely low in all the groups.

The presence of a lymphocytic infiltrate is a favorable prognostic indicator in some tumors, such as melanomas (Clemente et al. 1996), head and neck tumors (Uppaluri et al. 2008), and colon cancer (Jass 1986; Galon et al. 2006). The main mechanism of anticancer immunity is cell-mediated through the action of cytotoxic T lymphocytes (CTL) CD8+ and of Natural Killer (NK) cells, which occur in the inflammatory infiltrate surrounding tumors. The peritumoral CD57+ NK cells are significantly more numerous in KA when compared with SCC, suggesting that an early deficit of local immunity has already occurred. This deficit would lead to a failure in the mechanism that limits or impedes malignant transformation in the presence of potential carcinogenetic factors, such as UV rays or HPV infections. Therefore, we previously suggest that KA regresses when the local immunity is optimal, but it progresses to SCC if there is deficit of local immunity, such as, for example, when CD1a+ and CD57+ cells are low in number and/or functionality, even in patients that overall are immunocompetent (Cabibi et al. 2011). It is likely that Hsp60 plays a key role in this suggested mechanism of failure of local immunity that allows malignization of a relatively benign lesion, and if so, therapeutic measures ought to target also the chaperonin.

In addition, our findings open the door to investigations on the interplay between Hsp60 and CD1a cells in the mechanism of malignant transformation in KA and possibly other tumors. Elucidation of this interplay and its role in carcinogenesis might very well lead to the design of therapies centered on this chaperonin (Cappello et al. 2013). These therapies should consist of, for instance, direct administration of Hsp60 and/or boosting its anti-tumor potential by chemical compounds specifically designed to modify the chaperonin's

interactions and functions and direct them against progression of carcinogenesis. Our hypothesis of synergism between CD1a and Hsp60 in the progression from KA to SCC should boost interest in these tumors as models for studying the interaction mechanism between skin cancer and immunity.

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Compliance with ethical standards

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Conflict of interest The authors do not have any conflict of interest to disclose.

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