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Expression of 60-kD Heat Shock Protein Increases during Carcinogenesis in the Uterine Exocervix

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Key Words
Low-grade squamous intraepithelial lesion · High-grade squamous intraepithelial lesion · Squamous cervical cancer · Koilocyte · Chaperonin

Abstract
Objectives: The aim of the present study was to determine the presence and expression of the 60-kD heat shock protein (HSP60) in the dysplasia-carcinoma sequence in the uterine exocervix and to evaluate its diagnostic and prognostic significance. ***Methods and Results:*** We performed Western blot and immunohistochemical analyses on biopsies from 40 cases, consisting of 10 normal exocervical biopsies, 10 low-grade squamous intraepithelial lesions (L-SIL), 10 high-grade squamous intraepithelial lesions (H-SIL) and 10 cancerous exocervices (G2 grade). The immunohistochemical results were quantified by computer-assisted image analysis. Western blot analysis showed that HSP60 was undetectable in normal tissues and that there was a gradual increase of protein expression from L-SIL to carcinoma. Immunostaining for HSP60 was negative in normal tissue and positive in basal and parabasal layers of L-SIL epithelium; H-SIL were markedly stained in all layers of epithelium, and carcinomas showed an even stronger positivity. The increasing expression correlated with the malignancy grade. Finally, koilocytes were mostly negative in L-SIL and positive in H-SIL. ***Conclusions:*** The increasing degree of expression of HSP60 from L-SIL to carcinoma and the different intraepithelial distribution between L-SIL and H-SIL could be used as a new diagnostic tool. Moreover, HSP60 could have a role in cervical carcinogenesis.

Introduction

Exocervical cancer, the leading cause of death in women until 50 years ago, has become the 8th most common cause of female mortality in the last decades [1]. It is always preceded by precancerous lesions, which can exist at a noninvasive stage for as long as 20 years [1]. Early diagnosis and precise gradation of dysplasia are fundamental steps in establishing a correct prognosis. For this reason, many pathologists have focused their attention on the identification and classification of preneoplastic lesions of the uterine exocervix. Recently, the 'Bethesda classification' was established [2], which includes only

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two entities: low- and high-grade squamous intraepithelial lesions (L-SIL; H-SIL).

The heat shock protein (HSP) family is a class of proteins, highly preserved throughout evolution, which act mainly as molecular chaperones in protein folding. HSPs mediate a wide range of intracellular activities, and their high expression can be induced by a variety of cellular stresses. In particular, the 60-kD HSP (HSP60) is a molecular chaperonin localized in the outer mitochondrial membrane and, less frequently, in extramitochondrial sites [3]. Previous studies have pointed out the involvement of different HSPs, including HSP60, in carcinogenesis [4, 5]. Indeed, HSPs play important roles in cell cycle regulation and tumoral proliferation [6]. Furthermore, the possibility that the expression of HSPs may be a prognostic factor in cancer has recently attracted strong interest [7].

The aim of the present study was to analyze the expression of HSP60 in exocervical preneoplastic lesions (L-SIL and H-SIL), comparing them to normal and cancerous tissues, to investigate the possible role of this chaperonin in exocervical carcinogenesis.

Materials and Methods

Specimen Collection

Normal, dysplastic and cancerous tissues were collected, in a prospective manner, by punch biopsy at the Institute of Gynecology and Obstetrics of the 'Policlinico P. Giaccone' of the University of Palermo, Italy. Diagnoses were performed on 7-micra formalin-fixed, paraffin-embedded, HE-stained sections. The cervical dysplasias were histologically classified, following the Bethesda system, as L-SIL or H-SIL. We analyzed 10 different biopsies from L-SIL and 10 biopsies from H-SIL, as well as 10 normal exocervical biopsies and 10 G2 squamous cell carcinomas (SCC). A small specimen of each case was frozen for Western blotting analysis. Moreover, immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissues. Finally, the immunopositivity of HSP60 in the epithelium of uterine exocervix was quantified by a computer-assisted image analysis system, as described below.

Western Blot Analysis

20 μg of total cell extracts to each lane and a protein marker (Kaleidoscope prestained standard, catalog No. 1610324, Bio-Rad, Hercules, Calif., USA) were separated by electrophoresis on denaturing 10% polyacrylamide slab gel (SDS-PAGE) and transferred to nitrocellulose membrane (Nitrocell Paper, catalog No. 1620115, Bio-Rad). After 1 h at room temperature with a blocking buffer (5% low-fat dried milk in TBST: 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 0.1% Tween-20) under gentle shaking, the membranes were incubated with anti-HSP60 primary antibody (Sigma, St. Louis, Mo., USA, monoclonal mouse, clone LK1, catalog No. H4149) at a dilution of 1/500 overnight at 4°C. After washings, the membranes were incubated with HRP-conjugated secondary antibody (anti-mouse, 1/10,000, catalog No. 31432, Pierce, Cheshire, UK) for 1 h at room temperature with shaking and the specific binding was detected using a chemiluminescent substrate (SuperSignal West Pico Chemiluminescent Substrate, catalog No. 34080, Pierce) for autoradiography.

Immunohistochemistry

Immunostaining by the streptavidin-biotin complex method (LSAB2 kit peroxidase, catalog No. K0677, DAKO Corporation, Carpinteria, Calif., USA) was performed, using a primary antibody against HSP60 (Sigma, catalog No. H4149, 1/500) and isotype-matched control on 5-micra formalin-fixed, paraffin-embedded sections. After incubation for 10 min with serum-free protein block (DAKO, catalog No. X0909), the primary antibody was added to the sections. Nonimmune mouse serum was substituted for negative controls. Aminoethylcarbazole was used as the developing chromogen (DAKO, catalog No. K0677). Hematoxylin aqueous formula (DAKO, catalog No. S2020) was used for counterstaining.

Quantitative Analysis

The expression of HSP60 in the epithelium of uterine exocervix was quantified by computer-assisted image analysis (Colourvision 1.7.6, Improvision, Coventry, UK). For each biopsy, the entire epithelium in two nonserial sections was systematically assessed on the basis of red, green and blue color balance. At the beginning of each session, the image analysis system was standardized using the same section of exocervical mucosa stained for HSP60 to ensure reproducibility of the analysis. The digitized image of the standard section was used to interactively sample an example of the positive staining and the system was then allowed to select all the pixels of the same red, green and blue color balance (i.e. positive staining) within the image. The area of the epithelium was then delineated interactively and the percentage of positive staining within the epithelium was determined; the color balance and percentage staining value were recorded for future sessions. At the beginning of each subsequent session, the image analyzer was calibrated using this section and adjusted to within ± 5% of the original pixel reading. Once the system had been set up using the 'standard' slide, the test sections were analyzed using the same parameters. An observer who was unaware of the clinical group from which the biopsy specimen was derived performed measurements of HSP60 expression. Each tissue section was analyzed on two separate occasions by the same observer; means of the duplicate observation data were analyzed using the Mann-Whitney U test. $p < 0.05$ was considered significant.

Results

Morphological Characterization of the Tissue

Normal exocervices (NE) showed a squamous epithelium, with epithelial cells stratified in basal, parabasal, intermediate and superficial layers. L-SIL were characterized by thickening of the epithelium, and koilocytosis was frequently present (8 cases of 10) in the intermediate layer of epithelium. Nuclear atypia was commonly minimal and the mitotic index was very low [2–3 figures or less in 10 high-power fields (HPF)]. H-SIL showed thickness of the epithelium, and koilocytes were always present in the

middle and upper portions of epithelium. Rare bizarre or abnormal mitoses were found and the mitotic index was commonly moderate (4–10 figures in 10 HPF). Finally, islets of pleomorphic cells with large nuclei, conspicuous nucleoli and individual cell keratinization characterized the SCC. Nuclear atypia was frequent and the mitotic index was high (more than 10 figures in 10 HPF).

Expression of HSP60 in Human Uterine Cervices

Western blot results revealed a gradual increase of HSP60 expression in L-SIL, H-SIL and SCC (fig. 1). By contrast, HSP60 was undetectable in normal exocervical biopsies.

Immunostaining for HSP60 was negative in NE, showing a negligible positive signal in only 1 of 10 examined specimens (1–2 positive basal cells) (fig. 2A). L-SIL was

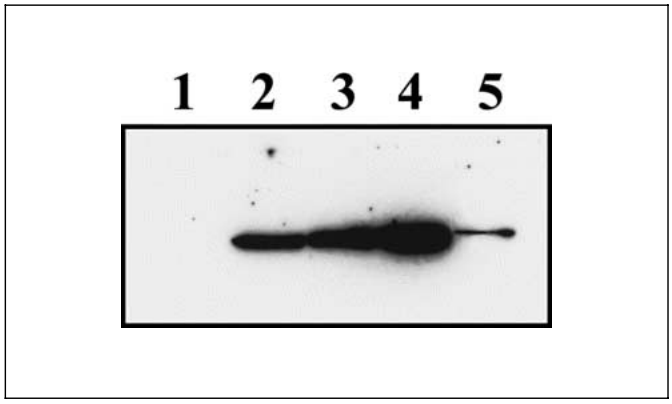

Fig. 1. Immunoblotting results. Lane 1: normal exocervical biopsies; lane 2: L-SIL; lane 3: H-SIL; lane 4: carcinoma; lane 5: marker. Increasing positivity for HSP60 from L-SIL through H-SIL to carcinoma is evident, while normal tissue is negative.

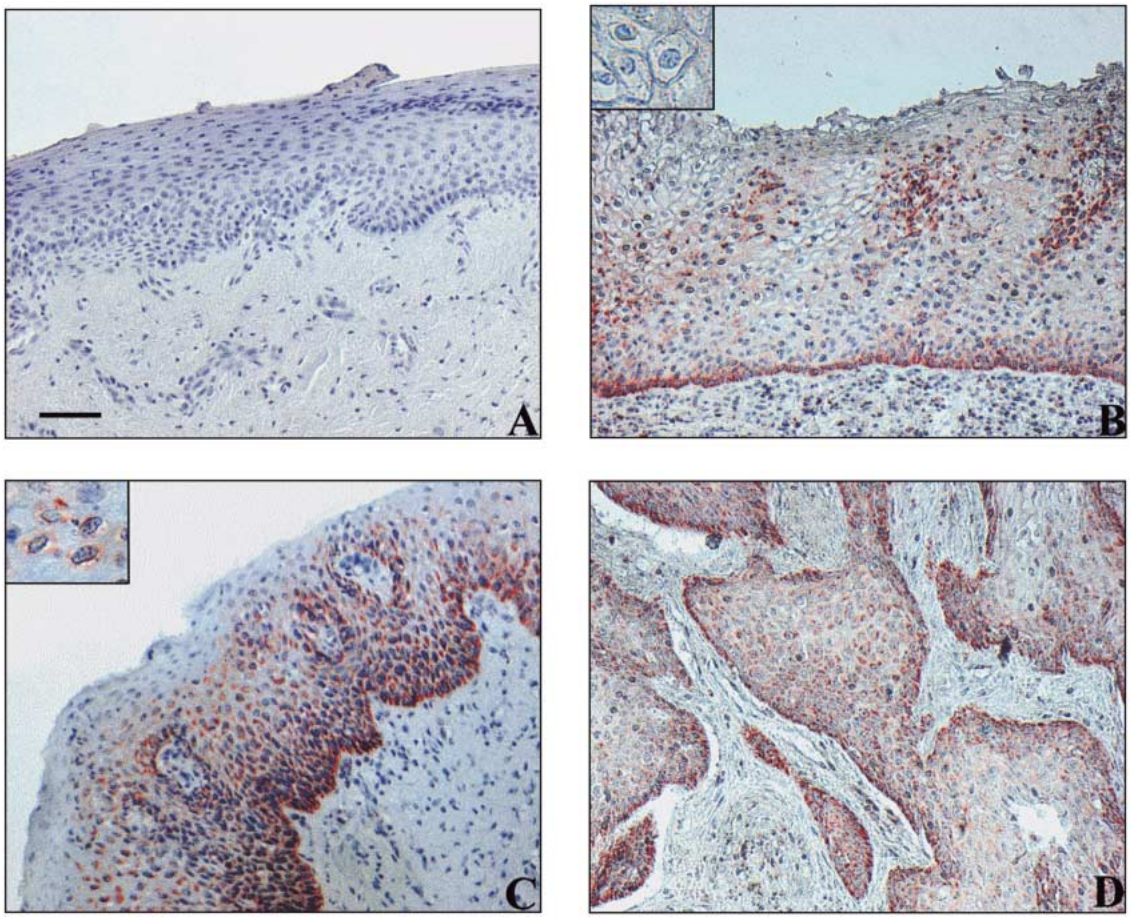

Fig. 2. Immunohistochemistry for HSP60. A In normal tissue, HSP60 was negative. B L-SIL: HSP60 positivity is localized mostly in basal and parabasal epithelial layers. Inset: negative koilocytes. C H-SIL: HSP60 is positive in all epithelial layers. Inset: positive koilocytes. D Carcinoma: islets of HSP60-positive cells. Bar: 50 μm.

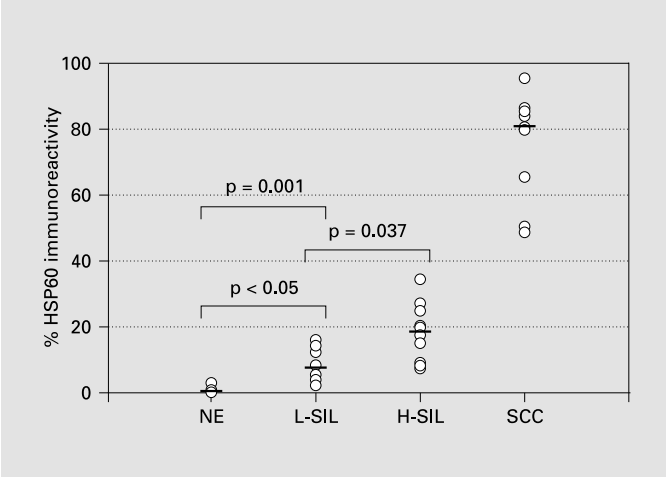

Fig. 3. Computerized image analysis showed that the level of HSP60 expression in NE, L-SIL and H-SIL differed significantly. SCC presented the highest expression of HSP60. The figure shows the percentage of HSP60 immunoreactivity. Each data point is a mean of duplicate observations obtained for each individual donor; the bars represent the median. Data were analyzed using the Mann-Whitney U test.

moderately positive for HSP60; in particular, basal and parabasal layers presented a cytoplasmic positivity, while HSP60 was scarcely present in the lamina propria (fig. 2B). Moreover, koilocytes were commonly negative in L-SIL (fig. 2B, inset). By contrast, H-SIL showed an intense cytoplasmic staining in all the epithelial layers (fig. 2C); in addition, the lamina propria showed occasional positive stromal elements, while koilocytes were commonly positive for HSP60 (fig. 2C, inset). Finally, SCC showed a strong positivity for HSP60 (fig. 2D), although interposed stromal cells were commonly negative, with a negligible number of positive stromal elements. Nuclear staining was not present in any of the biopsies.

Computerized image analysis revealed that the level of HSP60 expression in the uterine exocervical epithelium in L-SIL differed significantly from that in NE [percentage of epithelial staining, median (range): 8 (2–16.0) vs. 0.6 (0–3.0), $p < 0.05$]. Moreover, HSP60 expression in epithelium of H-SIL was significantly increased [18.3% (7.2–34%)] when compared to both L-SIL ($p = 0.037$) and NE ($p = 0.001$). Finally, the alteration of the morphological architecture in SCC (islets of tumor with interposed stroma) did not permit comparison of the HSP60 expression with that in the other conditions. However, HSP60 in SCC was always very high [80.3% (48.2–95%)] (fig. 3).

Discussion

Numerous subjective criteria are employed by pathologists to characterize SIL, but in some instances, the diagnosis is made by 'experience'; these 'instinctive diagnoses may not be reproducible' [2]. Changes in epithelial thickness or in nuclear density, conspicuous hyperchromasia, cell arrangements and differences in size and in staining of intermediate and superficial cells are some of the subjective criteria at the disposal of pathologists to diagnose an L-SIL. Moreover, evaluations of epithelial immaturity, nuclear atypia and inflammatory cellular changes, as well as regularity of nuclear spacing, preservation of nucleoli and absence of marked variation in nuclear size, are the main tools to distinguish an H-SIL.

The possibility of developing new diagnostic tools is therefore considered of great importance in all fields of surgical pathology. A number of antibodies against several keratins, CEA and blood group antigens have been tested to better characterize tumoral differentiation and invasion. Moreover, the cell proliferation index [8], lack of detection of human papillomavirus (HPV) DNA [9], stromal infiltration by S100 protein-positive Langerhans' cells [10] and expression of c-erbB-2 [11], ras oncogene [11] and Tn antigen [12] have been found to be related to an unfavorable prognosis in squamous cervical carcinoma. Recently, Klaes et al. [13] showed that $p16^{INK4A}$ was overexpressed in dysplastic and neoplastic cells of the cervix, allowing the sensitive and specific identification of dysplastic cervical cells in tissue sections or in cervical smears.

Experimental evidence concerning the role of HSPs in cell cycle regulation, DNA damage and carcinogenesis is continuously growing [6]. Numerous data support the hypothesis of an upregulation of HSP expression in different tumors, suggesting that HSPs are novel markers of malignant tumors [14]. Indeed, enhanced expression of HSP60 has been reported in breast carcinoma [15], myeloid leukemia [16] and ovarian [17] and prostatic carcinoma [18]. In particular, HSP60 overexpression has been found in both early and advanced prostate cancer, as well as in malignant prostate cancer cell lines, when compared to nonneoplastic prostatic epithelium [18]. Moreover, in another study, 10 of 23 osteosarcomas showed positivity for HSP60, but the level of expression did not correlate with clinical parameters [4]. By contrast, HSP60 expression was associated with a significantly better prognosis in 47 of 247 ovarian cancers [5].

Recently, high titers of HSP60 were detected in 3 of 29 patients with premalignant lesions of the oral cavity [19].

Moreover, the expression of HSP60 was altered during tongue carcinogenesis. Indeed, dysplastic lesions of the tongue stained for HSP60, while SCC were negative for this marker [20]. The cause of this different expression is not yet clear. In addition, immunocytochemical studies have shown the presence of HSP60 in other cellular sites, such as the cell surface, in various tumoral cells [21]. HSP60 could have the role of molecular chaperone in these sites as well as other unknown functions, e.g. mediating specific tumor signals [3, 21]. Finally, HSP60 has been shown to be expressed on the plasma membrane of human pancreatic carcinoma cells [22], and this expression could have a prognostic and diagnostic significance.

In this study, we found increasing expression of HSP60 from L-SIL through H-SIL to SCC. In particular, the circumscribed positivity for HSP60 in basal and parabasal layers of L-SIL, compared to the overexpression of this marker in H-SIL epithelium, if confirmed in a larger series, could have a diagnostic significance.

We also found a very low number of HSP60-positive koilocytes in L-SIL, compared to their frequent positivity in H-SIL. This evidence could help to discriminate between these different entities. the presence of koilocytes in the uterine exocervix is often considered a marker of HPV infection by pathologists [23]. HPV is known to contribute to the initiation of squamous carcinoma of the cervix, and it has been demonstrated that different HPV serotypes are associated with a different malignancy grade of the carcinoma [23]. Therefore, further studies determining HPV serotypes in exocervical biopsies could elucidate a potential correlation between HSP60 expression and the involved HPV serotype.

Computerized image analysis revealed an increase in the levels of HSP60 in the 'dysplasia-carcinoma sequence'. We can therefore postulate that HSP60 is involved in cancerous progression in the uterine exocervix. As a consequence, this different expression could have not only diagnostic importance but also a prognostic significance. Indeed, the expression of another HSP (HSP70) has already been suggested as a prognostic factor in cervix cancer [17], as it plays an important role in tumor proliferation. Moreover, HSP70 could also be implicated in the determination of the tumor grade [17]. In particular, Park et al. [24] found frequent HSP70 positivity in uterine cervical cancer, especially in the early stages; moreover, they analyzed clinicopathologic characteristics, expression of p53 and estrogen receptor and HPV infection status in their sequence of carcinomas, and found that these did not significantly correlate with HSP70.

The overexpression of HSP60 in the dysplasia-carcinoma sequence of the exocervix could also permit us to postulate that this chaperonin might have a different role in exocervical carcinogenesis from that in mitochondria regeneration during normal cell proliferation. Although normal exocervical squamous epithelium continuously regenerates its layers by mitosis of basal cells, HSP60 in normal specimens was generally under the antibody detection threshold in both Western blot and immunohistochemical analyses. By contrast, L-SIL and H-SIL, as well as carcinomas, showed a high expression of this protein. As a consequence, we hypothesize that the overexpression of HSP60 in these preneoplastic lesions could be the result of either an upregulation of this protein or the accumulation of a nonfunctional HSP60, but the exact mechanisms are still not clear.

In conclusion, HSP60 was shown to be involved in cervical carcinogenesis. Moreover, the different expression of HSP60 in L-SIL, H-SIL and carcinoma could be a new diagnostic tool, together with the evaluation of intrakoilocytic positivity. In addition, the relationship between HPV infection and HSP60 overexpression needs to be confirmed. Further studies on the overexpression of HSP60 could lead to the establishment of modern strategies for early diagnosis and prognosis of preneoplastic lesions of the uterine exocervix.

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