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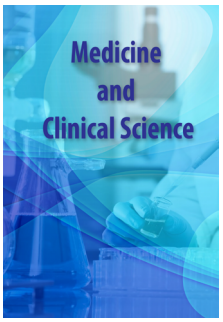

Differential expression of heat shock proteins in cutaneous squamous cell carcinoma

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Heat Shock Proteins; HSP; cutaneous squamous cell carcinoma; skin

Abstract

Cutaneous squamous cell carcinoma (CSCC) is a malignant tumor originating from keratinocytes in the epidermis, which seriously affects people's life safety. Heat shock proteins (HSPs) are molecular chaperones that help maintain protein stability and are involved in tumor activities. However, there is currently no systematic study of HSPs in CSCC. In this study, we used qPCR and WB to explore the expression of 14 HSPs at mRNA and protein levels in human immortalized keratinocytes HACAT cells, human CSCC cell lines COLO-16 and SCL-1 cells, and collected human CSCC samples for histological verification. Eventually, we found that the expression levels of different HSPs in CSCC were different. HSPB6, HSPB8 and HSPB11 showed no dysregulation in CSCC. HSPB4, HSPB5, HSPB7, HSPB10 and HSP40 were significantly upregulated in CSCC cells, while HSPB1, HSPB9, HSP60, HSP70, HSP90 and HSP100 were significantly downregulated. In summary, we validated the expression of 14 common HSPs in CSCC, which may lay the foundation for the study of HSPs in CSCC.

Introduction

Cutaneous squamous cell carcinoma (CSCC) poses a formidable health threat with its increasing incidence and mortality. Previously attributed primarily to cumulative sun exposure, the rise in CSCC rates is now known to also stem from an aging population as well as frequent use of tanning beds [1,2]. CSCC emerges from actinic keratoses, precancerous lesions in sun-damaged skin that evolve into invasive CSCC over time if left untreated [3]. Unlike most skin cancers that rarely metastasize, CSCC can spread aggressively into nearby tissues and gain access to lymph vessels and blood vessels, allowing tumor cells to colonize distant organs [4]. Once CSCC invades the dermis, the prognosis worsens considerably even with treatment. While superficial CSCC can be cured in over 95% of cases with surgery alone, more advanced tumors typically require a combination of surgery, radiation therapy, topical chemotherapy, and systemic therapy to have a reasonable chance of remission [5]. However, therapeutic options for patients with metastatic or advanced CSCC remain limited, highlighting the urgent need for more effective interventions against this highly morbid and potentially fatal disease.

Heat shock proteins (HSPs) constitute an important class of molecular chaperones found in prokaryotic and eukaryotic cells. HSPs are primarily classified into six major families based on their approximate molecular weight: HSP110, HSP90, Hsp70, Hsp60, Hsp40, and small HSPs [6,7]. Mainly expressed under stressful conditions, HSPs are protective proteins that aid in protein folding, stabilize protein structure, facilitate protein transport, and promote the degradation of misfolded proteins [8]. In these ways, HSPs help cells avoid damage from stress [9,10].

Currently, a large number of studies have found that HSPs are involved in the activities of tumors by adjusting oxidative stress [9,11], apoptosis [12] and autophagy [13] of tumor cells. For example, HSP40 is involved in the development of colorectal cancer [14], HSP27 induces resistance of pancreatic cancer cells to gemcitabine [15], targeting HSP90 can be used for the development of anti-cancer drugs [16]. However, there is a lack of systematic research on HSPs in CSCC.

In this study, we explored differential expression of 14 common HSPs in CSCC cells and keratinocytes. We further verified these findings in human CSCC samples. By investigating HSP expression in CSCC, we aimed to gain deeper insights into the relationship between HSPs and CSCC pathogenesis and establish a foundation for developing new therapeutic strategies against this disease.

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Materials and methods

Ethics approval

The Ethics Committee of The First Affiliated Hospital of Chongqing Medical University gave approval for this study. All patient signed informed consent form prior to the tumor removal surgery, and all experiments conformed to the norms of the Declaration of Helsinki. Informed consent was not applied to this study since it was retrospective.

Cell lines and cell cultures

Human cutaneous squamous carcinoma cell lines COLO-16 and SCL-1 cells and the immortalized keratinocytes HaCaT cells were obtained from National Collection of Authenticated Cell Cultures (SIBCB, China). The cells were incubated in a 37 ℃ incubator with 5% CO2; and were cultured in Dulbecco's Modified Eagle Medium (Procell, China), containing high sugar DMEM, 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin Solution, and 1% Alanyl-glutamine.

Patients

A total of 10 CSCC samples were collected from the tissue bank of the Department of Dermatology, The First Affiliated Hospital of Chongqing Medical University. The samples were selected from January 2019 to January 2020, along with complete clinicopathological data, and the patient's baseline data were listed in Table 1.

RT-qPCR

The mRNA-specific amplification primers for each target gene were purchased from Takara (Takara, Japan). Total RNA was extracted from the samples with SteadyPure Quick RNA Extraction Kit (Accurate Biology, China) and detected by a NanoDrop 2000 Spectrophotometer (Thermo Fisher, USA). cDNA was generated using RT Master Mix for qPCR (gDNA digester plus) (MCE, USA) and then amplified with the SYBR Green qPCR Master Mix (No ROX) (MCE, USA). After 40 cycles of amplification, the ΔΔCT equation was used to calculate the results. The sequences of primers were listed in Supplementary Table 2.

Western blot

The collected cells or tissues were lysed in RIPA Lysis Buffer (Solarbio, China) added with Protease and Phosphatase Inhibitor Cocktail (Beyotime, China). BCA Protein Assay Kit (Beyotime, China) was used to measure the protein concentration. The protein samples were transferred from the post-electrophoretic SDS gels onto nitrocellulose membranes. The membranes were blocked with 7% skim milk in TBST for 1 hour and subsequently probed with the primary antibodies overnight in a shaker at 4 ℃. On the second day, the membranes were incubated with the corresponding peroxidase-conjugated secondary antibody and then detected by Enhanced Chemiluminescence (Thermo Fisher, USA). The relative band intensity was quantified using ImageJ software (National Institutes of Health, USA). Antibodies: HSP20 (1:1000, A9091, Abclonal), HSPF1 (1:1000, A5504, Abclonal), HSP27 (1:1000, A11156, Abclonal), β-actin (1:1000, AC006, Abclonal).

Table 1. *Baseline Characteristics of Patients (n = 10)*

Variables	Mean (±SD) or count
Age	69 (± 9)
Sex	
Male	6
Female	4
Sites	
Exposure sites	7
Non-exposure sites	3
Breslow thickness	
Grade I (<3mm)	2
Grade II (3~6mm)	5
Grade III (>6mm)	3

Table 2. *Sequences of primer.*

Name	Sequence
HSPB1	F: CCCACCCAAGTTTCCTCCT
	R: GGCAGTCTCATCGGATTTTG
HSPB4	F: GGGTGTCTGTCTTCCTTTGCTTCC
	R: TCTCCTGGCTGCTCTCTCAAACC
HSPB5	F: CCAGGATGAACATGGTTTCATC
	R: ACAGGGATGAAGTAATGGTGAG
HSPB6	F: CCATCCTGGACACTGCCTTGATAAC
	R: GAGGGTTAGGGTAGTGCTGGTAGG
HSPB7	F: GAGCATGTTTTCCGATGACTTT
	R: TGATGTCTTCAGGTGAGAAGTC
HSPB8	F: TGGCAAACATGAAGAGAAACAG
	R: GGAAAGTGAGGCAAATACTGTC
HSPB9	F: GTAACACCTTCTCCAACGAGAG
	R: CTTCATTTGGAAACCGTCTCTG
HSPB10	F: CGACTTGTATATGCACCCCTAT
	R: GCTTCTCATCTTCTATGGCTCT
HSPB11	F: CAAAATGAAGAAATTGTGGCCG
	R: TGGCAAAACCCCATCTTTACTA
HSP40	F: AGTTCAAGGAGATCGCTGAGGC
	R: GCTGAAAGAGGTACCATTGGCAC
HSP60	F: TGCCAATGCTCACCGTAAGCCT
	R: AGCCTTGACTGCCACAACCTGA
HSP70	F: GACCTGCCAATCGAGAATCAGC
	R: CTGCGTTCTTAGCATCATTCCGC
HSP90	F: TCTGCCTCTGGTGATGAGATGG
	R: CGTTCCACAAAGGCTGAGTTAGC
HSP100	F: ACACCCTTGGATTATGCCCGAG
	R: GATGTGCTCCTTTAGTCGCTGC

Immunohistochemistry and hematoxylin-eosin staining

Tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sliced for hematoxylin-eosin staining and immunohistochemical staining. The immunohistochemical positive rate was quantified using ImageJ software (National Institutes of Health, USA). Antibodies: HSP27 (1:100, A11156, Abclonal), Ki-67 (1:100, A20018, Abclonal).

Statistical analyses

All experiments were repeated more than 3 times, Results were expressed as the mean ± standard error of the mean and analyzed using GraphPad Prism 9.3 software (La Jolla, USA). Student t-test was used for analyzing the differences between the two groups, and one-way analysis of variance (ANOVA) was used for analyzing the differences between three or more groups. P-value < 0.05 represents a significant difference.

Results

The mRNA expression of different HSPs in CSCC were different

HACAT cells, an immortalized human epidermal cell line, were selected as the normal control group. Two human CSCC cell lines, COLO-16 and SCL-1, were chosen as the CSCC groups. We detected the mRNA levels of 14 common HSPs in these three cell lines to explore differences in expression at the mRNA level between CSCC cell lines and keratinocyte cell lines. We found that among these HSPs, HSPB6, HSPB8, and HSPB11 exhibited no significant difference in expression between CSCC cell lines and HACAT cells (Figure 1A). Compared with HACAT cells, the expression of HSPB4, HSPB5, HSPB7, HSPB10, and HSP40 were significantly up-regulated in CSCC cell lines (Figure 1B), while the expression of HSPB1, HSPB9, HSP60, HSP70, HSP90, and HSP100 were significantly down-regulated in CSCC (Figure 1C).

The protein expression of HSPs in CSCC was consistent with mRNA level

Based on our findings at the mRNA level, we aimed to further verify the results at the protein level. Among the HSPs exhibiting no difference, low expression, and overexpression, we selected one HSP from each group for Western blot verification. We eventually chose HSP20, the protein expressed by HSPB6, HSPF1, the protein expressed by HSP40, and HSP27, the protein expressed by HSPB1, for Western blot verification (Figure 2A). We found that HSP20 expression showed no significant difference (Figure 2B). In contrast, HSPF1 expression was significantly increased in CSCC cells (Figure 2C), while HSP27 expression was significantly decreased (Figure 2D). The Western blot results were consistent with those at the mRNA level.

The expression of HSP27 in CSCCS was negatively correlated with the degree of malignancy

After obtaining results at the cellular level, we extracted 10 human CSCC samples for further verification. Through HE staining of CSCC tissues, we found regions II contained normal epidermal tissues adjacent to the tumor, while regions III exhibited typical CSCC tissues (Figure 3A). We then performed Ki-67 staining on the samples, finding Ki-67 expression was significantly higher in CSCC tissues than in normal epidermal tissues (Figure 3B). After HSP27 staining, we found HSP27 expression was significantly lower in CSCC tissues compared to normal epidermal tissues (Figure 3C). Evidently, the expression pattern of HSP27 in human tissue specimens mirrored that in the cell model. Moreover, Ki-67 staining revealed HSP27 expression was significantly negatively correlated with tumor malignancy.

Discussion

CSCC is the second most common skin cancer originating from malignant transformation of keratinocytes in the epidermis

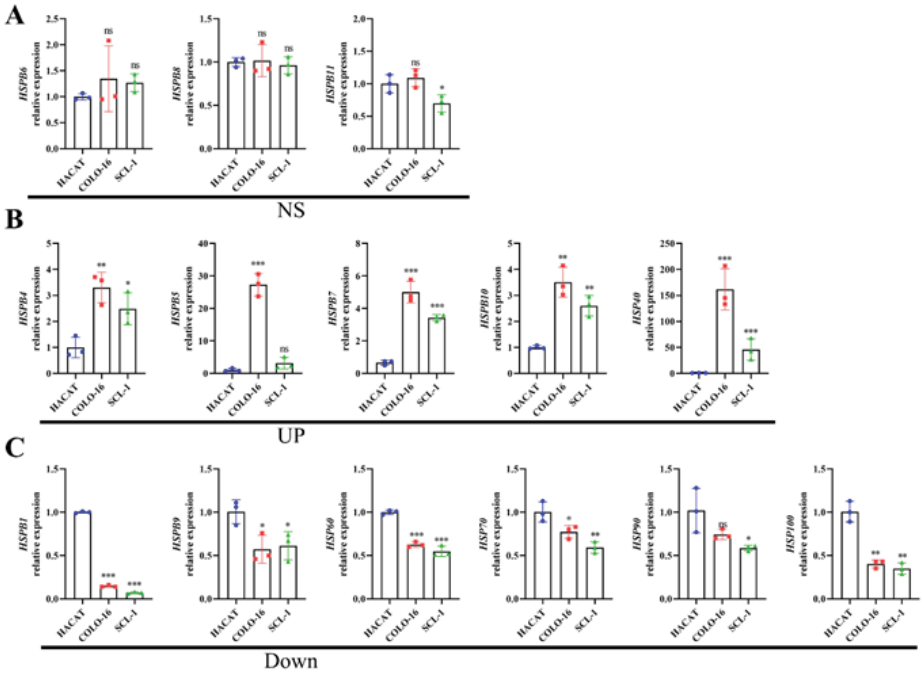

Figure 1. Changes in mRNA expression of different HSPs in CSCC.

*(A) qPCR results of HSPs with no significant difference in expression. (B) qPCR results of HSPs with up-regulated expression in CSCC. (C) qPCR results of HSPs with down-regulated expression in CSCC. N = 3, * P < 0.05, ** P < 0.01, *** P < 0.001, ns: no significant. HSPs: heat shock proteins.*

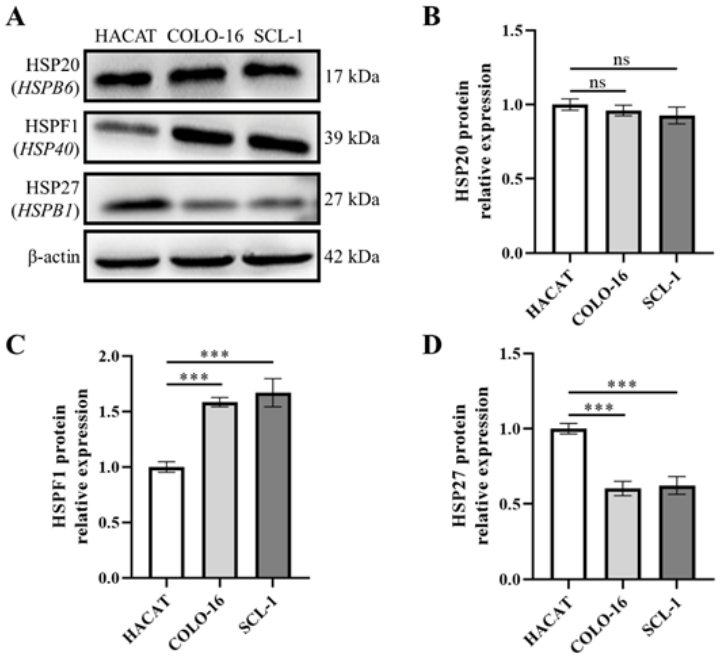

Figure 2. Changes in protein expression of different HSPs in CSCC.

*(A) WB results of different HSPs expressed in CSCCS. (B) Quantitative analysis of HSP20 protein band. (C) Quantitative analysis of HSPF1 protein band. (D) Quantitative analysis of HSP27 protein band. N = 3, * P < 0.05, ** P < 0.01, *** P < 0.001, ns: no significant. HSPs: heat shock proteins.*

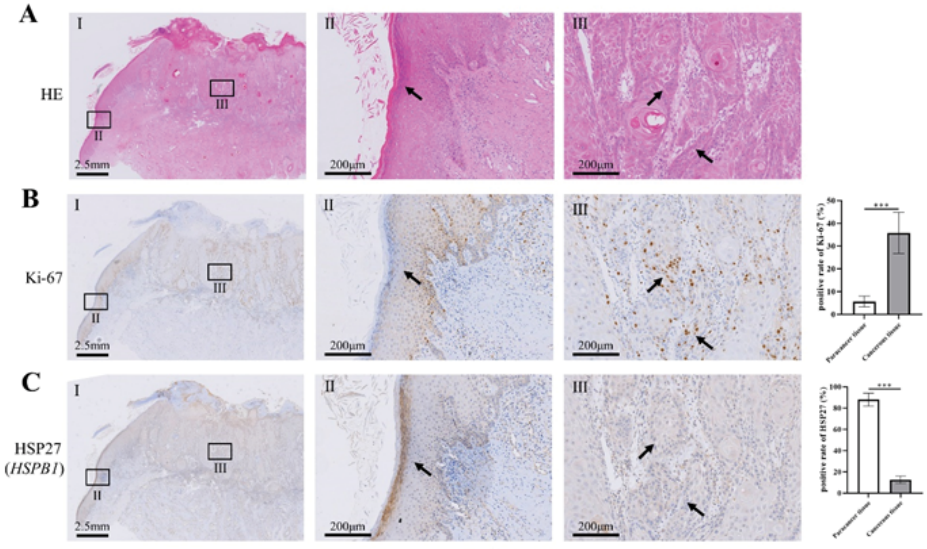

Figure 3. Sectioning and staining of CSCC tissues.

*(A) HE staining of sections of CSCC tissues. (B) Immunohistochemical staining and quantitative analysis of Ki-67 in CSCC tissues. (C) Immunohistochemical staining and quantitative analysis of HSP27 in CSCC tissues. N = 3, scale bars of image I= 2.5mm, scale bars of image II and III = 200μm, * P < 0.05, ** P < 0.01, *** P < 0.001, ns: no significant.*

[17]. CSCC poses a serious threat to human life and health. The main risk factor for CSCC is cumulative ultraviolet radiation exposure. In recent decades, due to population aging, increased sun exposure and use of tanning beds, the incidence of CSCC has increased sharply [18]. Although early CSCC can be cured alone by surgery, advanced CSCC usually requires a combination of modalities to maximize efficacy [19]. Therefore, it is necessary to continue to explore the changes of molecules in CSCC to understand and develop more effective measures to intervene in CSCC.

In this study, we examined the mRNA expression of 14 HSPs in CSCC cell lines and normal keratinocytes. We found that the expression of different HSPs in CSCC was different. Among them, HSPB6, HSPB8 and HSPB11 showed no dysregulation in CSCC. HSPB4, HSPB5, HSPB7, HSPB10 and HSP40 were significantly upregulated in CSCC cells, while HSPB1, HSPB9, HSP60, HSP70, HSP90 and HSP100 were significantly downregulated. The protein expression of HSPB1, HSPB6 and HSP40 was also verified to be consistent with the mRNA level.

However, the downregulation of HSP27, encoded by HSPB1, surprised us greatly. Currently, many studies confirm that HSP27 primarily promotes tumor progression and is highly expressed in most cancers, such as hepatocellular carcinoma [20], breast cancer [21], ovarian cancer [22], non-small cell lung cancer [23], and leukemia [24], leading to poor prognosis. To further verify the accuracy of our results, we examined HSP27 expression in human CSCC samples. We found significantly lower HSP27 levels in CSCC tumor tissues compared to adjacent normal tissues, consistent with Trautinger et al [25]. Moreover, HSP27 expression was inversely correlated with tumor malignancy. Tissues with higher Ki-67 expression, a marker of proliferation, showed lower HSP27 levels. This seems contrary to the high HSP27 expression reported in other cancers. In light of this contradictory finding, we considered the following possibilities.

We proposed the first idea, the "negative feedback" theory (based on the body's self-protective mechanism). It is well known that mutations in Ras, TP53, CDKN2A, and NOTCH1 genes are involved in the occurrence of CSCC [26]. These gene mutations may be early events of UV damage, paving the way for the development of CSCC [27]. The body offsets the malignant phenotype generated by gene mutations, thereby downregulating the protein HSP27 (a pro-tumor growth protein) to inhibit tumor growth. Due to the body's self-protective reaction, tumor tissues show a trend of low expression of HSP27. Unfortunately, the downregulation of HSP27 did not prevent the occurrence and development of CSCC.

The second idea is the "consumables" theory. In our experiment, the least HSP27 was detected in CSCC, because most of the HSP27 was consumed to supply tumor growth, which means the more malignant the tumor, the less HSP27. Whether the "negative feedback" theory and the "consumption" theory can reveal the low expression of HSP27 in CSCC still needs further practical research.

In summary, our study is the first to systematically evaluate HSP expression in CSCC. We have identified HSPs that are dysregulated at the mRNA and protein levels in CSCC cells and tissues. In particular, downregulation of HSP27 appears to be associated with CSCC development and severity. The study of HSP expression dysregulation in CSCC indicates that HSPs have the potential to become prognostic biomarkers or therapeutic targets for CSCC.

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Declaration of interest statement

All authors declare that no conflict of interest exists.

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