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Original Article

Expression of Hsp90α and cyclin B1 were related to prognosis of esophageal squamous cell carcinoma and keratin pearl formation

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Abstract: Hsp90α (heat shock protein 90α), one of the important molecular chaperones in cancer cell signal transduction, has been a new candidate target for cancer therapy. Cyclin B1, the client protein of Hsp90α, plays a key role as a mitotic cyclin in the G2-M phase transition during the cell cycle progression. However, the relationship between the level of HSP90α and cyclin B1, the location of Hsp90α and cyclin B1 in prognosis of esophageal squamous cell carcinoma (ESCC) has not been examined. Here, we demonstrate that the diagnostic significance of Hsp90α and cyclin B1 by immunohistochemistry and the association of Hsp90α and cyclin B1 expression in ESCC. In the specimens from 105 ESCC patients (81 stained with Hsp90α antibody by Immunohistochemistry, 65 with cyclin B1 antibody, and among them, 41 paired specimens were stained with Hsp90α and cyclin B1 respectively, and then checked for the correlation of the level and location of Hsp90α and cylcin B1. The positivity rate of Hsp90α and cyclin B1 expression were 96.3% (78 of 81) and 84.6% (55 of 65) respectively. Both of them, the expression levels are associated with the clinical pathological stage (Hsp90α, p=0.027; cyclin B1, p=0.007). No association was found between Hsp90α or cyclin B1 and gender, age, tumor location. As to TMN stage, there is no association with the level of Hsp90α, However, cyclin B1 expression is significantly related to tumor status (p=0.002). Interestingly, Hsp90α expression was negatively correlated to cyclin B1 expression (Gamma=-0.692, p=0.007) in the keratin pearls though there is a positive correlation in the other areas of tumor (Gamma=0.503, p=0.015), which suggest Hsp90α might play diverse roles in the cyclin B1 expression and cyclin B1 related cell cycle regulation in the different area of tumor. These findings demonstrated that the expression of Hsp90α, cyclin B1 protein is associated with tumor malignancy and prognosis for patients with human esophageal squamous cell carcinoma, and Hsp90α might be involved in cyclin B1 expression regulation and cell cycle regulation in keratin peal formation of ESCC.

Keywords: Hsp90α, cyclin B1, esophageal squamous cell carcinoma, keratin pearl, prognosis, immunohistochemistry

Introduction

Esophageal squamous cell carcinoma (ESCC) is a worldwide cancer, and ranks as 6th cancer-related death in the most common cancers [1]. China is one of the high incidence regions, and has the highest mortality in the world [2]. Nowadays advances in surgery and the introduction of new treatment of locally advanced carcinoma have been achieved, but the overall prognosis is still poor, with five-year survival rate less than 10% [3]. Thus, exploring the pathogenesis of ESCC, will contribute to find new targets for proper treatment of ESCC, and improve the prognosis.

Since the level of Hsp90 (heat shock protein 90) is increasing in many solid [4, 5] and hematological tumor such as hepatocellular carcinoma, acute leukemia et al [6, 7], it has been targeted for cancer therapy under clinical trials for years. Furthermore, Hsp90 is well known as the "cancer chaperone" [4], together with the cochaperones, it helps the client proteins to

maintain a correct conformation and is vital for degradation triage as well. Thus, it makes Hsp90 be a potential target to understand the pathogenesis of ESCC, and treat it in a multi-pathway-method [8].

Many genetic abnormalities have been revealed in ESCC, and altered expression of cell cycle regulators like cyclin D1, cyclin-dependent kinase (CDKs), cyclin B1 are related to the prognosis of the patients. As a client protein of Hsp90, cyclin B1 is a key protein in the control of cell cycle transiton from G2 to M phase [9]. It has been implicated that deregulated expression of cyclin B1 may lead to a disrupted control of cell growth and a malignant phenotype [10, 11]. Controversially, overexpression of cyclin B1 in patients with breast, colorectal, and hepatocellular cancers may induces a resistance to radiotherapy [12-14] and poor prognosis [15]. Confusingly, it has also been confirmed that after chemotherapy, those patients with follicular lymphoma could have a better prognosis which with higher levels of cyclin B1 [16]. Furthermore, Peters et al reported that there was not any association between cyclin B1 expression and prognosis after study with 56 invasive breast cancers [17]. In summary, these observations indicated that abnormal expression of cyclin B1 could be involved in neoplastic transformation, and the regulation of cyclin B1 level in tumor could be an attractive strategy for anti-proliferative therapy [14], but still need to be further confirmed in different tumors.

It is well known that the main function of Hsp90 is to help the folding of the denatured client proteins and the inhibition of Hsp90 will induce the degradation of the client proteins. However, the recent reports present that, the client protein of Hsp90, cyclin B1, slightly increased in presence of Hsp90 inhibitor Geldanamycin (GA) or in Hsp90 mutants, and Hsp90α is critical for the precise localizing cycliB1 to the mitotic spindle in Drosophila and human cells [18]. Also, it has been reported that treatment with Hsp90 inhibitors or heat shock induced an increasing mRNA level of cyclin B1 and the accumulation of cyclin B1 [19, 20]. All these indicate that Hsp90 might also be involved in to the cell cycle control of tumor cells, by regulation the level of cyclin B1. However, to our knowledge, there are no correlative immunohistochemical studies of Hsp90 and cyclin B1 expression in any neoplasm. Therefore, in the current study, we investigated expression levels of hsp90 and cyclin B1 in patients with esophageal squamous cell carcinoma (ESCC) by immunohistochemistry to gain insight into the effect of Hsp90 and cyclin B1 on the biologic behavior of esophageal tumors and to determine whether these expression levels have correlations and further clinicopathologic implications.

Materials and methods

Patients and tumor samples

One hundred and five patients with ESCC, including 65 males and 40 females with a mean age of 58.3 years (range, 31-83 years) were included in the study. Patients underwent surgical treatment in the Cancer Center of the Sun Yat-Sen University between March 2004 and March 2005. Surgical treatment consisted of resection of the esophagus with lymph node dissection without preoperative supplemental therapy.

Clinical staging and histopathological classification were based on the "The TNM Classification of Malignant Tumors (TNM) decided by the International Union Against Cancer". This project was approved by the ethics committee of the Cancer Center of the Sun Yat-Sen University and Southern Medical University.

Immunohistochemistry

In the specimens from 105 ESCC patients (81 stained with Hsp90α antibody by Immunohistochemistry, 65 with cyclin B1 antibody, and among them, 41 paired specimens were stained with Hsp90α and cyclin B1. 4 μm thick sections sliced from paraffin-embedded specimen were prepared on the glass slides. After deparaffinizing in xylene and washing in a graded series of ethanol, the sections were washed three times for 3 min with PBS. The sections were incubated with a blocking solution for 5 min to block any endogenous peroxidase activity and were placed in PBS. Then the sections were placed in the 0.01 M citrate buffer (pH 6.0) and heated at 121°C for 3 min in an autoclave oven. Then, endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 10 min. After washing in PBS 3X5 min, the sections were incubated with 1:300 rat anti-Hsp90α monoclonal antibody (9D2) (SPA-840, Enzo), Rabbit anti-cyclin B1 polyclonal antibody

Table 1. Clinicopathological parameters of the Patients with ESSC

Characteristic	Patients
ESSC	105
Gender	
Female	65
Male	40
Age	
Range	40
Median	58.3
Tumor site	
Medium	71
Lower	34
Tumor status	
T1	9
T2	30
T3	54
T4	12
Nodal Status	
N0	45
N1	60
Clinical Pathological Stage	
I	13
II	60
III	32

(H433, Santa Cruz) in 3% BSA/TBST for 90 min at room temperature. After washing in PBS 3X5 min, sections were subsequently incubated with a peroxidase-labeled anti-rat and anti-rabbit poly-antibody for 30 minutes at room temperature, respectively. Visualization was achieved using liquid DAB substrate-chromogen solution, with counterstaining with Mayer's hematoxylin, dehydrated, and mounted. Between steps, the slides were washed 3 times with water. Finally, Stained sections were photographed using Olympus IX51 light microscopes equipped with an Olympus DP-71 digital acquisition system. For negative controls, the primary antibodies were omitted and no antibody was used instead.

The results were scored with staining intensity and cell positive rate, as for no immunoreactivity is 0; weak immunoreactivity is 1; moderate is 2; strong is 3. Cell positive rate: <1% is 0; <10% is 1; <50% is 2; <80% is 3; >80% is 4, and the sum of the both scores is the final result. 0-2 for -; 3-4 for +; 5-6 for++; >7 for +++ [21]. Five fields were photographed for each slide, and at least 500 tumor cells for each sample were counted to generate the raw data. Then the raw scores were summarized as the final average scores. All slides were scored by two independent investigators. Discordant results were reevaluated jointly to reach a consensus.

Statistical analysis

Statistical association between Hsp90α, cyclin B1 expression and various clinicopathological factors was determined using the χ^2 test and the correlations were present by the gamma coefficient. The endpoint of follow-up was the date of death or the final contact till Aug. 2013. Survival duration was calculated from the month of surgery. For survival analysis, survival data of 72 patients stained with Hsp90α antibody by Immunohistochemistry, and data of 45 patients with cyclin B1 antibody were available and analyzed. Since the total number of available cases were limited, we classified the -/+ to negative, ++/+++ to positive to generate the survival curves. The cumulative survival rates were calculated using the Kaplan–Meier methods, and the statistical significance of differences was determined using the log-rank test. All statistical tests were operated with the program SPSS 16.0 and $p<0.05$ was considered statistically significant.

Results

Clinicopathological characteristics

Specimens from 105 patients were fixed in 10% formalin and embedded in paraffin. The type of cancer was histopathologically diagnosed by reviewing hematoxylin and eosin-stained sections. Patients were classified according to the TNM system, describing size and range of the tumor, the lymph nodes and the metastasis status. All patients were without evidence of distant metastases at the time of first diagnosis. The clinicopathological characteristics of patients with ESSC are summarized in **Table 1**.

Immunohistochemical staining of HSP90α in ESSC

The higher intensity of Hsp90α staining signals were observed predominantly in the cancer cells, locate in both of cytoplasm and nucleus

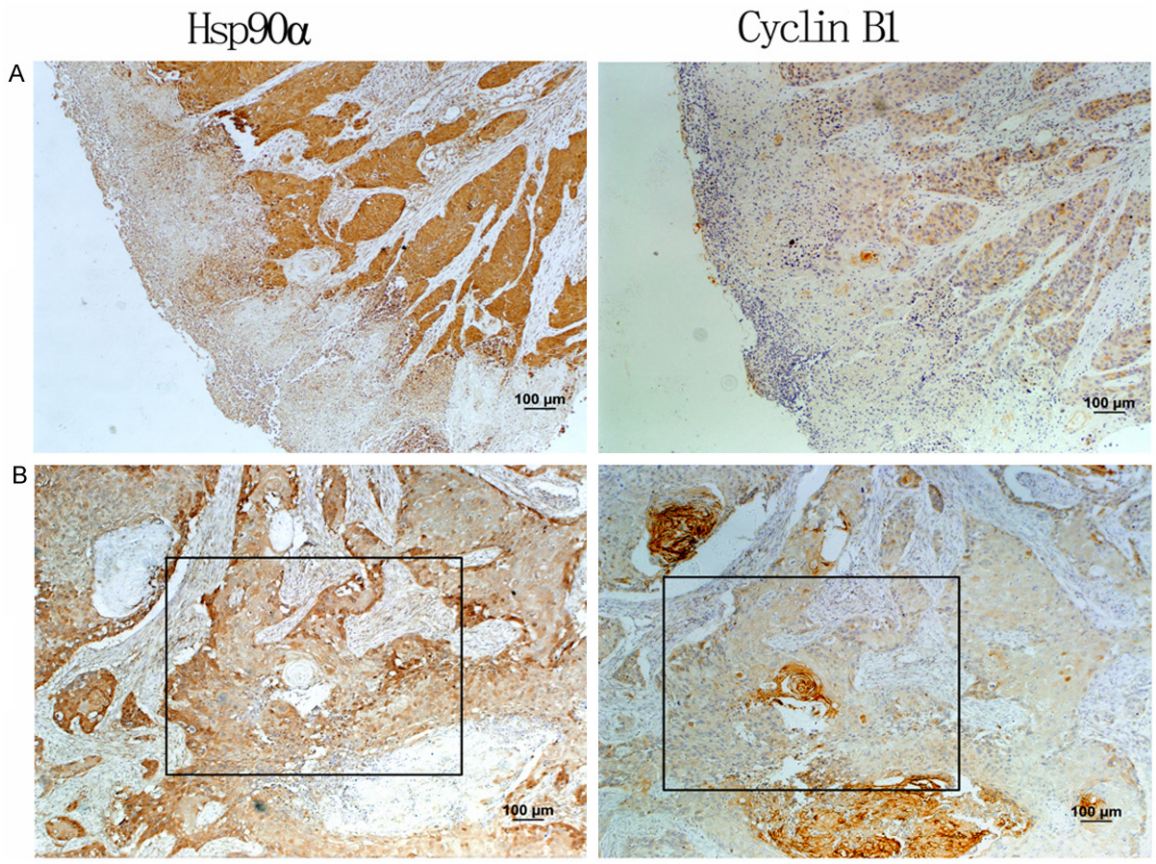

Figure 1. Immunohistochemical staining of HSP90α and cyclin B1 in ESSC. The higher intensity of Hsp90α staining signals were observed predominantly in the cancer cells, locate in both of cytoplasm and nucleus. As higher intensity of cyclin B1 staining signals scattered in the carcinoma cells. In poor differentiated ESCC, the intensity of immunohistochemical staining of Hsp90α and cyclin B1 is higher than poor differentiated ESCC. A. Poor differentiated ESCC; B. Well differentiated ESCC.

(**Figure 1**). In well differentiated ESCC, which characterized with more keratin pearls, the intensity of immunohistochemical staining of Hsp90α is lower than the poor differentiated ones (**Figure 1A** and **1B**). Furthermore, the cells at the edge of well-differentiated tumors demonstrated higher expression of Hsp90α than the other cells. All these indicate Hsp90α might be necessary for the tumor growth and invasion. Among 81 cases of ESSC in surgical specimens, Hsp90α was significantly overexpressed in 78 cases (96.3%) (**Table 2**). No association was found between overexpression of Hsp90α in patients with age, gender, tumor site, tumor status, and nodal status. However, significantly increased Hsp90α expression was more frequently observed in later clinical pathological stage than other stages (p=0.003, **Table 2**).

Cyclin B1 expression is related to the differentiation of ESSC

The localization of cyclin B1 was observed by immunoreactivity in the cytoplasm and nucleus of cancer cells, and poor differentiated ESCC was with higher intensity and more nucleus distribution of cyclin B1 (**Figure 1**). In 65 surgical specimens of ESSC, cyclin B1 was overexpressed in 55 cases (84.6%). No association was found between overexpression of cyclin B1 in patients with age, gender, tumor site. As to TMN stage, cyclin B1 expression is significantly related to T stage (p=0.002). Furthermore, the expression of cyclin B1 (the locations of cyclin B1 in the keratin pearls were excluded) was association with the clinical pathological stages (p=0.001, **Table 2**).

Table 2. Relationship between Hsp90α and Cyclin B1 overexpression and clinical variables in ESCC

Parameters	Hsp90α				Cyclin B1			
	-/+	++	+++	*p* value	-/+	++	+++	*p* value
Gender								
Female	7	23	26	0.97	14	19	11	0.99
Male	3	11	11		7	9	5	
Age								
30-	1	5	8	0.11	4	6	3	0.11
50-	7	24	27		13	19	7	
70-	2	5	2		1	3	6	
Tumor site								
Medium	7	26	26	0.82	17	25	11	0.45
Lower	3	8	11		4	3	5	
Tumor status								
T1	1	5	3	0.4	5	2	1	0.02
T2	4	11	10		7	8	4	
T3	4	14	21		8	10	6	
T4	1	4	3		1	8	5	
Nodal Status								
N0	2	14	13	0.78	12	13	7	0.409
N1	8	20	24		9	15	9	
Clinical Pathological Stage								
I	2	6	2	0.03	5	1	1	0.01
II	6	19	17		12	15	6	
III	2	9	18		4	13	9	

Table 3. Relationship between Hsp90α and Cyclin B1 overexpression in tumor cells

Hsp90α	Cylin B1			Gamma	*p* value
	-/+	++	+++		
-/+	5	1	1	0.503	0.015
++	2	6	5		
+++	3	7	11		

Correlation between Hsp90α and cyclin B1 expression

We investigated whether there is a correlation between Hsp90α and cyclin B1 expression. 41 paired specimens were stained with Hsp90α and cyclin B1 antibody respectively, and the matched areas were observed and photographed one by one.

The result showed the Hsp90α expression was positively correlated to cyclin B1 expression (Gamma=0.503, p=0.015, **Table 3**) in cancer cells except the center of keratin pearl area.

The keratin pearl is a character of well-differentiated ESCC, which is further confirmed by HE staining. Whereas the strong staining signal of cyclin B1 always appear in the center of the keratin pearls, the Hsp90α expression levels are low and even became negative in the center of them (**Figure 2**), which indicates the Hsp90α might be involved in the regulation of cyclin B1 expression and degradation. The statistical analysis was done with the 44 matched keratin pearls, and the result showed there was a negative correlation between the expression of Hsp90α and cyclin B1. (Gamma=-0.692, p= 0.007, **Table 4**).

Survival analysis

Kaplan-Meier survival analysis was used to determine survival rate with respect to the expression of Hsp90α and cyclin B1. The result showed that higher Hsp90α expression were significantly associated with the worse prognosis (p=0.002) and cyclin B1 showed the same tendency. (p=0.002, **Figure 3**).

Discussion

In this study, we found that the expressions of Hsp90α and cyclin B1 in ESCC were obvious in poor-differentiated tumor cells and significantly higher in later clinical pathological stages (p<0.05), suggesting that they might be the potential biomarkers in the progression of ESCC. It is well known that Hsp90α and cyclin B1 expressions are tightly associated with the development and progression of tumors [22, 23]. Since our conclusion drew from the ESCC specimens of operation, without any preoperational therapy, that could explain the diversity result different from the tissue collected after chemotherapy [16], and more data from ESCC patients should be collected and analyzed to further confirm this.

Current studies have indicated an association of HSPs with cancer and demonstrated their

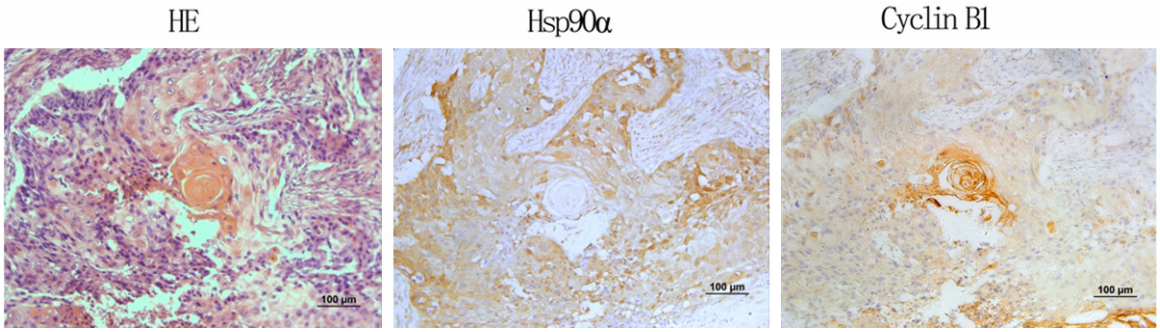

Figure 2. The opposite staining of Hsp90 and cyclin B1 in Keratin pearls. The keratin pearl area from **Figure 1** was further confirmed by HE staining. The staining signal of cyclin B1 is significantly high in the center of the keratin pearl, whereas the Hsp90α expression level is low or negative.

Table 4. Relationship between HSP90α and cyclin B1 overexpression in keratin pearls

Hsp90α	Cylin B1		Gamma	*p* value
	-	+		
-	5	20	-0.692	0.007
+	11	8		

important roles in cancer biology [8, 24]. In view of the key roles of the molecular chaperones in the tumor growth and differentiation, we hypothesize it may exert a major impact on the clinical outcome of patients [25-27]. It is said that there is 'oncogenic stress' results in the inducible expression of heat shock proteins that assist in the recovery from stress either by repairing damaged proteins (protein refolding) or by degrading them, which evokes a DNA damage response network that delays or prevents cancer at the beginning of tumorigenesis, thus restoring protein homoeostasis and promoting cell survival [28, 29]. Thus, this study demonstrated that the expression of Hsp90α could be a prognostic tool to predict the aggressiveness of ESCC.

Cyclin B1, an essential cell cycle regulator, as a prognostic factor in esophageal squamous cell carcinoma has been examined earlier [10, 30]. Moreover, they reported patients overexpressed cyclin B1 showed better outcome after chemotherapy compared with patients that had lower expression levels of cyclin B1. Also in a study from Anna J. suggested that the overexpression of cyclin B1 increased apoptosis that induced by chemotherapy in human brain tumor cells and HeLa cells [31, 32]. However, our result was the opposite; the lower expression of cyclin B1 predicted the better prognosis, similar to some earlier viewpoints [33, 34]. All these suggested the dynamic change of cyclin B1 expression might be linked to the different stage of ESCC or chemotherapy, and the delicate change of cyclin B1 is critical for the overcome of ESCC.

In this study, we describe a novel type of positively correlated regulation of Hsp90α and cyclin B1 expression in ESCC tumor cells. This is an initial study to indicate that the expression of Hsp90α and cyclin B1 were associated with the malignancy of ESCC. The match number of patients in advanced stages and the total number of tumor tissues with Hsp90 staining intensity and cyclin B1 staining intensity still need to be increased and be weighted more in further study.

Keratin pearl which is a type of structure, sometimes seen with squamous cell carcinoma. A keratin pearl means a local accumulation of trapped squamous epithelium. Though the mechanism of keratin pearl formation remains unclear, some evidence supports that oxidative stress [35], might be the reason to induce the cells locate in the center portion of well differentiated ESCC to death. In our experiment with oxidative stress inducer H_2O_2 treated well-differentiated esophageal carcinoma cell line TE-1, although there was no obvious increase of Hsp90α, cyclin B1 accumulation, cell cycle arrest and proliferation inhibition (data unpublished, Figure S1) were observed.

However, how might Hsp90 affect cyclin B1 expression? Some reports indicated that Hsp90α inhibitions affected the redox status

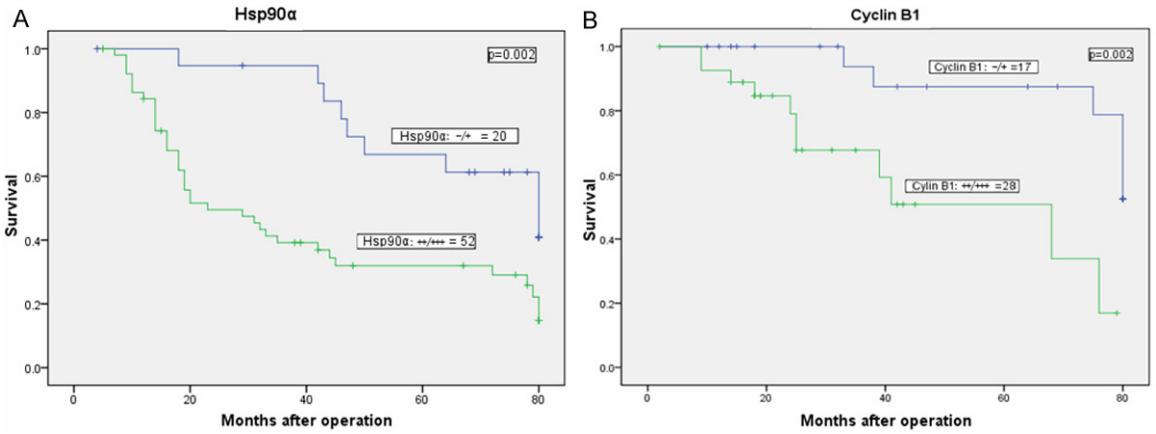

Figure 3. Kaplan-Meier's survival curves with the expression of Hsp90α and cyclin B1. Kaplan-Meier survival analysis was used to determine survival with respect to expression of Hsp90α and cyclin B1. We classified the -/+ to negative ++/+++ to positive, respectively. A. Overall survival curves of cases with ESCC according to Hsp90α expression. B. Overall survival curves of cases with ESCC according to Cyclin B1 expression. The survival in ESCC with Hsp90α expression was significantly lower than that in ESCC without Hsp90α expression ($p<0.01$), the same as cyclin B1 ($p<0.01$).

and induces cyclin B1 accumulation on related G2/M cell cycle arrest in tumor cells [36, 37]. Cancer cells are normally under a hypoxia environment, and the center of the tumor nest could encounter a more sever hypoxia condition, combined with the high metabolism rate in the cancer cells, could induce a higher oxidative stress than normal cells. In certain melanoma cell lines which hypo-proliferated in such situation, presented lower hsp90 expression than others [38]. As Hsp90 is essential for eukaryotic cell viability and it chaperones more than 200 overexpressed client proteins in cancer, the lower expression of Hsp90 in the center of tumor nest of ESCC could be play a key role to induce fibrosis and keratin pearl formation. Since the abnormal change of cell cycle regulators induces cell cycle arrest, like our findings of sequestered cyclin B1 in the center of the tumor nest of ESCC, the elevated level of 14-3-3δ, another client protein of Hsp90, was found around cysts in hyperproliferative squamous differentiation tumor. Since loss of 14-3-3σ leads to increased proliferation and impaired differentiation in squamous cell carcinoma [39], and elevated levels of 14-3-3δ enforce a G2/M cell cycle arrest by sequestering Cdc2/cyclin B1 complexes in the cytoplasm and are required for a stable G2/M arrest after DNA damage [40]. This also support the cyclin B1 related keratin pearl formation could be linked to oxidative stress induced DNA damage response of squamous tumors. All these suggest, different extent of oxidative stress would affect Hsp90α function, especially the pathway of protein degradation triage, and sever ones eventually lead to the client proteins, like 14-3-3σ, cyclin B1, accumulation and cell damage.

The aim of the present study was to elucidate the clinicopathological significance of Hsp90 and cyclin B1 in ESCC by examining the expression level with Immunohistochemistry in the ESCC tissues. The most interesting finding is that there is a positive correlation between Hsp90α and Cyclin B1 expression in the ESCC tumor areas and by contrast, a negative correlation in the keratin pearl areas. Although the histopathogenetic process of keratin pearls formation is still poorly understood, the well-differentiated squamous cell carcinoma mostly companied with an acceleration of keratinization in squamous cell carcinoma (SCC) cells. This may represent one possible therapeutic avenue for ESCC [41]. Thus, the unique distribution of Hsp90α and cyclin B1 in the keratin pearl areas might indicate a potential role of Hsp90α in cyclin B1 regulation for cancer therapy.

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Disclosure of conflict of interest

None.

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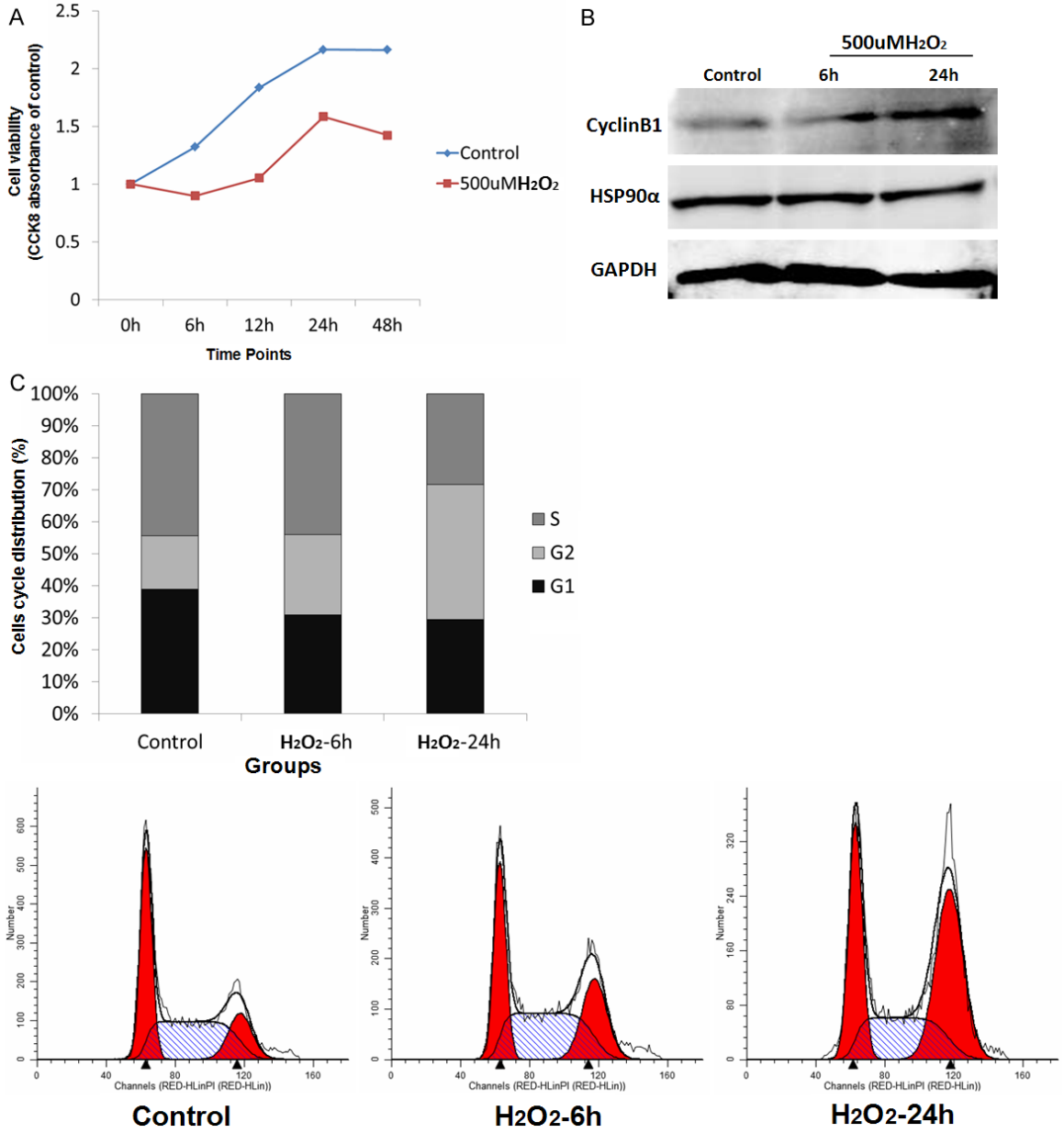

Figure S1. Oxidative stress inhibited the proliferation of well-differentiated esophageal carcinoma cell line TE-1 by G2/M arrest with decreased Hsp90α and cyclin B1 accumulation. A. 500 μM H_2O_2 inhibited TE-1 proliferation; B. Expression of Hsp90α slightly decreased, and cyclin B1 obviously increased; C. Flow cytometry results showed 500 μM H_2O_2 induced G2/M arrest.