Abstract

OBJECTIVES: Chronic Obstructive Pulmonary Disease (COPD) is accompanied by increased cellular stress and inflammation. Most of the Heat Shock Proteins (HSPs) have strong cytoprotective effects. The role of HSPs in COPD pathogenesis has not determined completely. We investigated the serum level of HSPs in COPD patients, smokers without COPD and healthy non-smoking controls. Also, we evaluated the relationship of HSPs with various parameters (inflammatory, oxidative, functional status, quality of life) in COPD patients.

MATERIAL AND METHODS: The levels of stress protein (HSP27, HSP70, HSP60, HSP90, CyPA), interleukin-6, C-reactive protein and malondialdehyde were measured in 16 healthy non-smoker, 14 smokers without COPD and 50 patients with stable COPD. Pulmonary function tests (PFT) and arterial blood gases parameters were measured. Health Related Quality of Life was evaluated and exercise capacity was measured with 6 minute walking test.

RESULTS: Only HSP27 levels was significantly higher in COPD patients when compared with both healthy non-smoker and smokers without COPD (for both, p< 0.001). There was a weak-moderate negative correlation between serum levels of HSP27 and PFT parameters and between HSP27 levels and PaO2. Serum levels of HSP27 showed a weak-moderate positive correlation with symptom, activity and total scores. Subjects evaluated only smokers without COPD and patients with COPD; HSP27 had an area under the curve (AUC) in the receiver operating characteristic (ROC) curve of 0.819 (0.702-0.935; 95% CI; p= 0.000).

CONCLUSION: Increased serum levels of HSP27 was found in COPD patients and our results showed sensitivity and specificity of serum HSP27 as diagnostic markers for COPD.

KEYWORDS: COPD, heat shock protein, oxidative stress, hypoxia

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INTRODUCTION

There is prominent inflammatory response and oxidant-antioxidant imbalance in Chronic Obstructive Pulmonary Disease (COPD). Also, persisting inflammatory reactions continue in COPD patients despite cessation of smoking. Cigarette smoking is the major risk factor for COPD. Cigarette smoke contains multipl free radicals and these toxic substances are believed to induce an inflammatory response by adversely affecting oxidant/antioxidant and protease/anti-protease balance in the lung. In fact, COPD don’t develop all smokers. The majority of long-term smokers do not develop COPD suggests that failure of compensatory mechanisms that protect the lung from reactive oxygen species (ROS) or xenobiotic materials contributes to development of the disease. The expression of antioxidant genes believed to be important in protection of the lung from cigarette smoke-induced injury. Recent studies indicate that a complex molecular cascade termed the “unfolded protein response” (UPR) plays an important role in the regulation of expression of a variety of antioxidant, xenobiotic metabolizing and pro- and anti-inflammatory genes [1].

Heat shock proteins (HSPs) are chaperones that catalyze the proper folding of nascent proteins and the refolding of denatured proteins. HSPs have a role either the renaturation or the destruction of damaged proteins under stressful conditions such as heat, bacterial or viral infections [2]. HSP27 was first reported to contribute to heat shock resistance; subsequently, its involvement in diverse protective mechanisms against toxicity mediated by aberrantly folded proteins or oxidative-inflammatory conditions has also been confirmed [3]. Under normal physiological conditions the synthesis of most HSPs is low. However, when organisms endure stress such as heat shock and inflammation, where protein damage is increased, certain HSP are induced and expressed at high levels [4]. Increased HSPs levels showed in COPD patients. HSPs, especially HSP60 may have a role in COPD pathogenesis and some HSPs might be used as possible...
serum markers for determining COPD in the smoking subjects [5,6]. To our knowledge, the role of HSPs in pathogenesis and diagnosis of COPD has been investigated in few studies.

The aim of our study was to investigate whether the serum levels of various HSPs are elevated in smokers without COPD and COPD patients and to determine the relationship between HSPs and several parameters (inflammatory, oxidative, functional status and quality of life) in COPD patients.

PATIENTS and METHODS

Subjects

This study was done between September 2012 and April 2013. This case control study included 80 patients with COPD and controls. Healthy non-smoker volunteers (n= 16), smokers without COPD (n= 14), patients with COPD (n= 50) were evaluated.

Control group, consisted of 16 healthy non-smoking subjects and 14 smokers without COPD, had normal pulmonary function parameters and they had not any lung disease. All subjects were selected with Stratified Random Sampling Method from amongst the hospital staff. The age and sex of the control subjects were similar to COPD patients.

Fifty stable COPD patients enrolled into the study and they were taken from a hospital respiratory out-patient clinic. COPD was diagnosed according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria [7]. In addition, the classification of airflow limitation severity was evaluated according to GOLD guidelines [7]. Patients with no evidence of an exacerbation for one month before study were accepted as clinically stable. Acute exacerbation as defined by GOLD, use of systemic steroids within the past 14 days, asthma, autoimmune diseases, lung cancer, known 1-antitrypsin deficiency and known cardiopulmonary co-morbidity were considered as exclusion criteria.

Ethical approval was obtained by the institutional review board (31.05.2012-09) and informed consent was obtained from each subject.

Age, gender and smoking history were asked and the body mass index (BMI) and pulmonary function tests (PFTs) was detected in all subjects. Six minute walking test (mwt), arterial blood gases (ABG) analysis were done in COPD patients and Health Related Quality of Life (HRQL) also evaluated in COPD patients.

Pulmonary Function Testing

The pulmonary function tests were done using a spirometry device (Ultima CPX 790705-205, St. Paul, MN, USA). The standard spirometric examination was conducted according to European Respiratory Society (ERS) criteria [8]. Forced expiratory volume in 1 s (FEV1) and forced vital capacity (FVC) are expressed as percentages of predicted values (FEV1 % pred and FVC% pred) according to the prediction equations of the ERS [8].

Health Status Measurement

HRQL was assessed in COPD patients using the Turkish version of St. George’s Respiratory Questionnaire (SGRQ) [9,10]. The questionnaire was applied to COPD patients by the same interviewers. The SGRQ has been used extensively for assessing quality of life in patients with COPD and several other chronic lung diseases [11]. It contains 50 items with 76 weighted responses that cover three domains: symptoms-distress due to respiratory symptoms, activity-disturbances of physical activity and impact-overall impact on daily life and well-being. In addition to the domain scores, there is also a total score [9]. The SGRQ is scaled from zero to 100 (with zero representing the best health-related quality of life).

Exercise Performance

Exercise performance was evaluated by the 6 mwt according to the American Thoracic Society Guideline [12].

Arterial blood gas measurement

Arterial blood gas samples of COPD patients were taken at rest, in a sitting position and in room air at the room temperature. Samples were measured by a blood gas analyse device (Rapid lab 348. Biobak., Chiron, Bayer Diagnostic, UK).

Measurement of serum HSPs, CRP, IL-6, CRP and MDA levels

Blood samples were collected between at 8.30-9.30 following 10-hours starvation. Serum was acquired after centrifugation and aliquots were kept frozen at -20°C until further testing. HSP27, HSP70, HSP60, HSP90, CyPA and interleukin-6 (IL-6) were determined using adapted enzyme-linked immunosorbent assay (ELISA) kits according to kits protocol. Levels of HSP27, HSP60, HSP70, HSP90, CyPA, and IL-6 were determined using adapted ELISA kits ([Boster Biological technology., Ltd. (Catalog no: EK0881), assaypro (Catalog no: EH5505-1), Hangzhou eastbiopharm co. Ltd. (Catalog no: CK-E11197), Hangzhou eastbiopharm co. Ltd. (Catalog no: CK-E11190), Hangzhou eastbiopharm co. Ltd. (Catalog no: CK-E90142), Boster immunoleader (Catalog no: EK0410), respectively] according to kits protocol.

The concentration of serum malondialdehyde (MDA) was determined by High-performance liquid chromatography (HPLC) using Immuchrom commercial kit (ImmChrom GmbH, Munich, Germany) according to kit protocol.

Serum levels of C-reactive protein (CRP) were routinely analyzed by the Central Laboratory at the hospital.

Statistics

Data were analyzed using the statistical package for the social sciences (SPSS) software statistical program. Results were given as median and 95% CI. A p value of < 0.05 was considered statistically significant. Statistical analysis was performed using Kruskal-Wallis test for multiple-group comparisons; Mann-Whitney U test was performed to test any observed differences for significance and results were interpreted according to Benferroni correction. Chi-square test was performed to compare gender distribution between
groups. Spearman’s correlation was used to assess non-parametric data. Receiver operating characteristic (ROC) curves were plotted to show sensitivity and specificity of the evaluated HSPs.

RESULTS

Age, gender and BMI were found similar between the patient population and control subjects (p> 0.05). Patient characteristics and PFt parameters were shown in Table 1. There was no statistically significant difference in the levels of HSP70, HSP90, HSP60 and CyPA between groups (p> 0.05) (Table 2). The serum levels of HSP27 were statistically higher in COPD patients than in both healthy non-smoker and smokers without COPD (for both p< 0.001) (Table 2, Figure 1A). There was no statistically significant difference in the levels of HSP27 between healthy non-smoker and smokers without COPD (p> 0.05).

When the HSPs evaluated according to classification of airflow limitation severity; 29 (58%) COPD subjects were GOLD I-II and 21 COPD subjects (42%) were GOLD III-IV. Statistically significant difference only were found for HSP27 between healthy non-smoker and COPD GOLD I-II (p< 0.01), healthy non-smoker and COPD GOLD III-IV (p< 0.001), smokers without COPD and COPD GOLD I-II (p< 0.05), smokers without COPD and COPD GOLD III-IV (p< 0.001), COPD GOLD I-II and COPD GOLD III-IV (p< 0.05) (Figure 1B).

There was no statistically significant difference in the IL-6 levels between groups (p> 0.05). The levels of CRP were statistically higher in COPD patients than in both healthy non-smoker and smokers without COPD (p< 0.001 for both) and the levels of MDA were significantly higher in COPD patients when compared to healthy non-smoker (p< 0.001) and smokers without COPD (p< 0.01) (Table 2).

The mean duration of disease was 6.00 ± 6.25 year, the mean PaO₂ was 63.35 ± 9.71 mmHg, PaCO₂ was 37.94 ± 5.90 mmHg, and SaO₂ was 91.27 ± 4.40%, the mean 6 mwt was 368.36 ± 112.10 m and the mean symptom score was 53.47 ± 24.51, activity score was 50.59 ± 22.37, impact score was 38.23 ± 22.89 and total score was 44.50 ± 22.03 in COPD patients (Table 3). The mean PaO₂ levels was significantly higher in COPD GOLD I-II patients (68.38 ± 7.17 mmHg) than COPD GOLD III-IV patients (56.41 ± 8.49 mmHg) (p< 0.001).

Table 1. Demographic characteristics of all subjects

<table>
<thead>
<tr>
<th></th>
<th>Healthy non-smoker</th>
<th>Smokers without COPD</th>
<th>COPD patients</th>
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<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td>Age (Year)</td>
<td>65.18 ± 4.72</td>
<td>66.00 ± 6.43</td>
<td>66.84 ± 7.39</td>
</tr>
<tr>
<td>Male /Female</td>
<td>14/2</td>
<td>14/0</td>
<td>46/4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.46 ± 2.17</td>
<td>24.70 ± 2.02</td>
<td>24.73 ± 4.37</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoker</td>
<td>16 (100%)</td>
<td>0</td>
<td>7 (14%)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>0</td>
<td>0</td>
<td>23 (46%)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>0</td>
<td>14 (100%)</td>
<td>20 (40%)</td>
</tr>
<tr>
<td>Pack-years</td>
<td>0</td>
<td>24.85 ± 5.64</td>
<td>35.76 ± 10.73</td>
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Lung function

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<tbody>
<tr>
<td>FEV₁ (pred %)</td>
<td>96.43 ± 5.09± b</td>
<td>90.42 ± 4.43b</td>
<td>54.70 ± 1.98</td>
</tr>
<tr>
<td>FVC (pred %)</td>
<td>92.93 ± 6.44b</td>
<td>87.78 ± 6.77b</td>
<td>69.18 ± 17.26</td>
</tr>
<tr>
<td>FEV₁/FVC (%)</td>
<td>87.25 ± 2.46± c</td>
<td>81.78 ± 2.96b</td>
<td>56.72 ± 1.08</td>
</tr>
</tbody>
</table>

COPD: Chronic obstructive pulmonary disease, FEV₁: Forced expiratory volume in one second, FVC: Forced vital capacity, BMI: Body mass index. Compared with group II: ± p< 0.01; ± p< 0.001. Compared with group III: ± p< 0.01.

Table 2. Serum levels of heat shock proteins, interleukin-6, malondialdehyde, C-reactive protein in all groups

<table>
<thead>
<tr>
<th></th>
<th>Healthy non-smoker</th>
<th>Smokers without COPD</th>
<th>COPD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSP 27 (pg/mL)</td>
<td>983 (662.67-2749.85)a</td>
<td>1370 (1042.65-1963.34)a</td>
<td>2679.50 (2474.63-3230.44)</td>
</tr>
<tr>
<td>HSP 70 (ng/mL)</td>
<td>151 (111.17-170.5)</td>
<td>101.55 (101.12-195.96)</td>
<td>92.05 (105.18-150.53)</td>
</tr>
<tr>
<td>HSP 90 (ng/mL)</td>
<td>3.41 (2.97-6.02)</td>
<td>3.1 (2.79-9.39)</td>
<td>3.06 (3.33-6.09)</td>
</tr>
<tr>
<td>HSP 60 (ng/mL)</td>
<td>1.71 (1.41-2.54)</td>
<td>2.31 (1.81-2.9)</td>
<td>2.02 (2.19-3.93)</td>
</tr>
<tr>
<td>CyPA (ng/mL)</td>
<td>0.87 (0.74-1.97)</td>
<td>0.8 (0.71-2.45)</td>
<td>0.84 (0.91-1.48)</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>65.4 (45.4-117.14)</td>
<td>66.25 (59.98-106.14)</td>
<td>60.9 (63.3-81.54)</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>0.1 (0.05-0.4)</td>
<td>0.04 (0.05-0.36)</td>
<td>0.9 (0.84-1.42)</td>
</tr>
<tr>
<td>MDA (µmol/L)</td>
<td>1 (0.67-1.07)</td>
<td>1.02 (0.96-1.04)</td>
<td>1.06 (1.04-1.07)</td>
</tr>
</tbody>
</table>

Compared with group III: ± p< 0.001; ± p< 0.01. Results were given as median and 95% CI.
Serum levels of HSP27 showed a weak to moderate negative correlation with FEV1, FVC and FEV1/FVC values (Respectively, r = -0.428, p < 0.01, r = -0.389, p < 0.01, r = -0.383, p < 0.01). Only weak to moderate positive correlation were found between serum levels of HSP60 and IL-6 levels (r = 0.327, p < 0.05). Serum levels of HSP27 showed a weak to moderate positive correlation with symptom, activity and total scores and duration of disease (respectively, r = 0.351, p < 0.05, r = 0.294, p < 0.05, r = 0.316, p < 0.05). There was a weak to moderate negative correlation between HSP27 and PaO2 (r = -0.367, p < 0.01). There was a weak to moderate positive correlation between HSP27 and duration of disease (r = 0.399, p < 0.01) (Table 4).

In addition, we evaluated diagnostic value of HSP27 because of increased HSP27 levels was found in COPD patients. Subjects evaluated only smokers without COPD and patients with COPD; HSP27 had an area under the curve (AUC) in the receiver operating characteristic (ROC) curve of 0.819 (0.702-0.935; 95% CI; p= 0.000). A HSP27 level of 2260 pg/mL was taken as the cut-off between smokers without COPD and COPD patients, HSP27 had a sensitivity of 78% and specificity of 70% (ROC curve) (Figure 2A,B).

**DISCUSSION**

Our study shows increased levels of HSP27 in COPD patients. But the levels of HSP27 levels were not significantly different between non-smokers and smokers without COPD. Also, the levels of serum HSP27 are significantly increased in both COPD GOLD I-II and COPD GOLD III-IV patients than control subjects. When the patient’s general health status was deteriorated, increased levels of HSP27 determined. The negative relationship was found between HSP27 levels and ABG and PFT parameters.

The reasons of increased release of HSPs into the extracellular environment are; the constant induction of inflammatory signals and upregulation of intracellular HSPs due to increased cellular turnover [5]. HSPs may be increased in several inflammatory disease. Elevated serum levels of HSP27 were reported in inflammatory disorders including acute coronary syndrome and chronic allograft nephropathy and increased HSP90 immunostaining was found in inflammatory regions of human atherosclerotic plaque [13-15]. They are highly conserved chaperone proteins that regulate the folding and processing of damaged proteins and...
HSP27 and HSP90 behaviors as a defensive factor against unfavorable stimuli such as heat shock and oxidative stress and it can modulate ROS and increases glutathione levels [21]. This ability results cytoprotective affects of HSP27. It is showed that HSP27 and 90 have a protective against oxidative stress [22]. The facilitator effect in the antioxidant defenses of increased HSP expression was already shown in healthy sedentary subjects [23]. HSP27 expression in smokers with or without COPD may be predominantly attributed to hypoxia and inflammation and they have protective effect in the lung cells against oxidative stress in smokers and COPD patients [20]. Increased levels of HSP27 in the lungs of smokers and especially smokers with COPD showed that increased levels of HSP27 is related with primarily oxidative stress and partly inflammation [20]. Increased serum HSP27 levels were found in subjectively healthy smokers who determined emphysema with HRCT without spirometric impairment [24]. These results shows that immune response caused by inhaled toxins in smokers’ make pulmonary changes in HRCT and cause decreased HSP27 levels into the pulmonary vascular network in COPD sensitive subjects and HSP27 increases after the development of radiological COPD even thought there was no functional impairment. We found increased serum HSP27 levels in COPD patients. There was no significantly difference in HSP27 levels between healthy non-smokers and smokers without COPD subjects. Therefore we think that increased serum HSP27 levels may not be directly associated with smoking and it can only be detected increased when COPD develops. But COPD patients had higher smoking index than smokers without COPD in our study. This may affect our data. However, oxidative stress due to smoking causes the secretion of proteins but increased serum HSP27 levels in COPD patients may ascribed to other contributing factors such as hypoxia and inflammation. Also, the mean MDA levels were significantly higher in COPD patients compared with healthy non-smokers and smokers without COPD, but we did not show any relationship between HSP27 and MDA levels as an indicator of oxidant system. Further studies must be done for determining the exact antioxidant role of HSP27 in COPD patients.

Elevated HSP27 levels were reported in inflammatory disorders and HSPs expression is low under physiological conditions [14]. But HSP27 levels are temporarily increased when stress events developed and later their concentrations are decreased by termination of the acute triggering. HSP27 levels increase only when its cytoprotective effects are necessary [25]. Contrary, a continuous increase in serum HSP27 levels parallel with disease severity was shown previously [5]. Augmentation of tissue destruction in late stages of COPD and systemic inflammation of COPD may cause a systemic spillage of HSP27 into the vascular bed. Similarly, we found a continuous enhancement in serum HSP levels with severity of airflow limitation and there was an increase in HSP27 levels when respiratory function decreased and duration of disease increased. It also supports the idea that HSP27 is related with the increased tissue destruction and systemic inflammation in COPD. The relationship between serum HSP27 levels and PFT parameters as well as duration of disease interpreted that serum levels of HSP27 may be useful predictor of severity of airflow limitation in COPD stages and evaluation of response to treatment. Furthermore, we think that in addition to the systemic inflammation of COPD, hypoxia can be a contributing factor on continuous increases of HSP27, because there was a prominent hypoxia in COPD GOLD III-IV patients.

Figure 2. (A) A cut-off level of HSP27 in smokers without COPD and COPD patients, (B) Receiver operating characteristic (ROC) curve indicating sensitivity and specificity of HSP27 to diagnose COPD in the Smoking study population.
Previous experimental studies have shown increased production of HSPs in response to anoxia, presumably to help stabilize/protect protein structure/function [26,27]. Responses of HSPs are organ specific [26,28]. There are a little data for the production of HSPs in the lung airway cells response to chronic hypoxia [29]. Increased HSP70 and HSP90 and unchanged HSP70 levels in lung tissue against chronic hypoxia were shown [29-31]. Consequently, the activation of heat shock response is important in stress-responsive pathways to long-term anoxic survival [32]. In our study, PaO2 negatively correlated with serum HSP27 levels. This interpreted that hypoxia is a prominent contributing factor on HSP27 levels in COPD patients.

The HSP levels are related with circulating levels of CRP and cytokines. Cytokines may increase the induction of HSPs and contrarily HSPs may decrease the release of cytokines [33]. Serum CRP and IL-6 levels were positively correlated with serum HSP levels [34]. The mechanisms of increased HSP expression due to inflammation are still not understood. In nuclear factor-IL-6 may have regulatory roles in HSP expression [35]. Different responses may be seen in HSP expression against cytokines, for example IL-6 levels increased the HSP90 levels but decreased HSP70 levels in peripheral blood mononuclear cells [36,37]. We only found a positive correlation between HSP60 and IL-6 but there was no correlation between HSPs and CRP levels. Because some of the HSP cover more than one gene, their inducible expression may be changed according to comment.

Previous studies showed that in generally increased HSP levels in COPD patients [6,20]. They may originate peripheral airways, lung interstitial cells or in other organs [5,6]. The role of extracellular HSP60 is unclear but some studies showed their pro-inflammatory effects in atherosclerosis [38,39]. Similar to Hacker’s et al study, we did not find that HSP60 have a role in the pathogenesis of COPD [5]. Furthermore, we did not find any difference in serum HSP70 and HSP90 levels between the groups. The differences between study results can be due to methodological differences and differences of subject characteristics (age, gender, smoking index and respiratory function). On the other hand, HSPs are paradoxical molecules. Intracellular HSPs have beneficial and protective roles but extracellular HSPs are signal molecules for the immune system and extracellular HSPs have a modulating effect to the secretion of pro-inflammatory cytokines [40]. The exact role of serum HSPs in COPD, determines of endogenous and exogenous trigger mechanisms has to be addressed in further studies.

To our knowledge, effect of HSPs on quality of life in COPD patients has not been examined until now. Molecular chaperone expression may induce with psychological stress and psychological stress induced HSP expression was shown in rats [41,42]. For this reason, we wanted to evaluate the relationship between SGRQ scores and HSPs. SGRQ symptom, activity and total scores were significantly associated with higher serum concentrations of HSP27 in COPD patients. These findings implicate that HSP27 may be a related factor on quality of life in COPD patients. On the other hand, several factors such as duration of the disease, disease severity and hypoxia were closely related to quality of life in COPD patients [43-45]. For this reason increased HSP27 levels may be related with decreased respiratory functions and hypoxemia on quality of life in COPD. Moreover, HSP27 may be used for the evaluation of functional status and prediction of disease severity because of HSP27 levels correlated with PFT parameters.

Proteomic analysis for determining of disease markers in COPD patients have been done previously. Serum levels of HSP27 and HSP70 may be a potential diagnostic marker and they can show disease severity [5]. Similarly these results, serum contents of HSP27 showed high sensitivity and specificity for diagnosis of COPD in our study. Because of the high sensitivity and specificity, HSP27 might be a suitable marker for diagnosis of disease according to our results.

In conclusion, the level of HSP27 was increased in COPD patients. Because smokers without COPD subjects had normal levels of HSP27 one can suppose that hypoxia is the effective factor rather than oxidant stress on serum levels of HSP27. In addition, HSP27 may be a marker of quality of life and functional status. Further investigations enrolling higher numbers of patients are needed to establish the role of HSP27 on COPD pathogenesis.


Conflict of Interest: No conflict of interest was declared by the authors.

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