Plasma Levels of Heat Shock Protein 90 Alpha Associated with Lung Cancer Development and Treatment Responses

Yuankai Shi¹, Xiaoqing Liu², Jiatao Lou³, Xiaohong Han¹, Lijian Zhang⁴, Qingtao Wang⁵, Baolan Li⁶, Mei Dong⁷, and Yinghong Zhang⁸

Abstract

Purpose: Altered expression of heat shock protein 90 alpha (Hsp90α) was associated with tumor development, progression, and metastasis. This study explored plasma levels of Hsp90α protein in patients with lung cancer and other controls to assess its diagnostic value and monitor treatment responses for patients with lung cancer.

Experimental Design: A total of 2,247 individuals were recruited and assigned into two cohorts as static and dynamic groups. ELISA analysis and confirmation of plasma Hsp90α protein levels for association with tumor stages and treatment responses, respectively, were performed.

Results: The average plasma levels of Hsp90α protein in patients with lung cancer were significantly higher than in healthy controls (P < 0.0001). Plasma levels of Hsp90α protein in patients with advanced lung cancer (stage III–IV) were higher than in patients with early-stage lung cancer (stage I–II; P < 0.001). Using a cutoff value of 56.33 ng/mL to separate lung cancer from other controls, the sensitivity and specificity reached 72.18% (95% CI, 0.695–0.749) and 78.70% (95% CI, 0.761–0.813), respectively. To confirm the different levels in the second cohort, plasma levels of Hsp90α protein showed a statistically significant difference between preoperative and postoperative patients in surgical patient groups (P < 0.0007). There was also a statistically significant difference between the disease progressive group and stable disease group, with regard to partial response after chemotherapy (P < 0.0001).

Conclusions: This study demonstrated that plasma Hsp90α protein levels are useful as a diagnostic biomarker in lung cancer and predict the responses of patients with lung cancer to chemotherapy. Clin Cancer Res; 20(23); 6016–22. ©2014 AACR.

Introduction

Lung cancer is the most common cause of cancer mortality in the world. In 2013, it was estimated that there were over 1,600,000 incident cancer cases and 580,350 cancer deaths in the United States, accounting for 28% of lung cancer deaths in men and 26% of lung cancer deaths in women (1–3). Despite the development and use of molecular targeted drugs, which have improved treatment modalities for lung cancer, the overall 5-year survival rate still remains at 15% to 20% (1, 2). Lung cancer is usually diagnosed at the advanced stages of disease, which makes curable surgery impossible. Lung cancer also frequently metastasizes to the lymph nodes and distantly, which makes chemotherapy and radiotherapy ineffective. Although there are many techniques of clinical diagnosis for lung cancer, such as X-ray, CT scan, PET-CT, fine-needle biopsy, and bronchoscopy, they all have certain limitations. Therefore, to improve the outcome of these patients, noninvasive and rapid diagnostic methods with high sensitivity and specificity are urgently needed. As such, both scientific and clinical researchers put most of their efforts into discovery of novel biomarkers and molecular targets. A pertinent example was the development and use of...
molecular targeted drugs (icotinib and gefitinib) for treatment of a subset of patients with lung cancer with EGFR mutation (4). However, for diagnostic markers in lung cancer, there are a number of biomarkers being assessed in the clinic, though almost all of these have low sensitivity or specificity (5).

Our research has focused on heat shock proteins, which are a group of proteins induced by heat shock or stress and play an important role in normal protein folding. Such proteins maintain active protein conformation and intracellular disposition for growth and survival of cells (6, 7). Heat shock proteins are named according to their molecular weight. Hsp90 has 2 isoforms, Hsp90α and Hsp90β. Only Hsp90α is exposed to the extracellular space, and the presence of Hsp90α on the cell surface was associated with tumor development and progression, especially with cancer metastasis (8, 9). For example, a previous study showed that levels of plasma Hsp90α protein in patients with cancer were significantly associated with cancer progression and metastasis, as well as with age, tumor volume, and estrogen receptor (ER) expression. This indicates that Hsp90α is an independent marker for tumor diagnosis and prognosis (10). However, to the best of our knowledge, there is currently no single study with a large clinical sample size reporting diagnostic and prognostic significance of plasma Hsp90α levels in lung cancer. In this study, we recruited more than 2,000 people who had been diagnosed with either lung cancer or were healthy controls, or had benign lung disease for ELISA assessment of plasma levels of Hsp90α protein. The results demonstrated that plasma Hsp90α protein levels are useful as a diagnostic biomarker of lung cancer and prediction of the responses of patients with lung cancer to chemotherapy. Thus, detection of Hsp90α protein could help to monitor the treatment responses.

Materials and Methods

Patients and plasma samples

This study was a multicenter prospective clinical study with the participation of eight different hospitals. Two cohorts of patients or controls were recruited for this study from these eight hospitals between September 2008 and March 2012. The first cohort (defined as the static group) included 1,046 patients with lung cancer, 592 healthy controls, 344 patients with noncancerous lung lesions, and 17 patients with benign lung tumor for evaluation of Hsp90α protein levels in plasma samples. We chose noncancerous lesions from patients with lung and benign lung tumor, to increase the specificity. Stages for patients with lung cancer were classified according to the American Joint Committee on Cancer Classification (the 7th edition). The eligibility criterion for recruiting these lung cancer cases was histologically confirmed lung cancer. Furthermore, no adjuvant radiotherapy was administered to any of these patients. We collected plasma (EDTA-K2 anticoagulant) samples from all participants and then stored them at –20°C, until use.

The second cohort was defined as the dynamic group and included 248 consecutive patients with newly diagnosed lung cancer, of which 79 patients received surgery and 169 patients underwent chemotherapy. For surgical patients, we collected plasma samples 3 days before surgery, 3 to 7 days after surgery, and 3 days after clinical evaluation (imaging), respectively. For chemotherapy patients, who were treated with pegylated recombinant human endostatin or placebo combined with paclitaxel/carboplatin for four cycles. After four cycles, patients who were evaluated as stable disease (SD) would continue the treatment with pegylated recombinant human endostatin or placebo until disease progression. We collected baseline plasma samples (before the first chemotherapy cycle) and subsequent plasma samples (any day between each treatment cycle interval until the fourth cycle). Two milliliters of whole blood was collected in EDTA-K2 anticoagulant tubes and centrifuged at 3000 × g for 10 minutes at room temperature. Plasma samples were stored at –20°C, until use. We evaluated treatment efficacy after the second and fourth cycles of chemotherapy using CT scan. Based on the results of CT scan, the patients were grouped into partial response (PR), progressive disease (PD), and SD and then compared to the change in Hsp90α levels in each patient.

This study was approved by the Institutional Review Board (IRB) of Cancer Institute/Hospital of Chinese Academy of Medical Sciences and Peking Union Medical College and by the other seven hospitals. Written informed consent was obtained from each individual or patient.

ELISA detection of plasma levels of Hsp90α protein

Before testing our study samples, we first validated the accuracy and precision of the ELISA Kit for Hsp90α protein (Yantai Protgen Biotechnology Development Co., Ltd, Yantai, China). Specifically, the intra-assay and interassay accuracy and precision were determined by assessing quality control (QC) samples at low, medium, and high concentrations in five replicates, in three independent batches. The deviation of accuracy and precision from the nominal concentration was required to be < ±15%, whereas the low QC samples should not exceed 20%. The intra-assay accuracy and precision for spiked samples ranged from 1.1% to 8.9% and 4.1% to 11.3%, and the inter-assay accuracy...
and precision ranged from −1.1% to 1.7% and 1.3% to 5.8%, respectively, indicating that the ELISA Kit had good accuracy and precision.

For the ELISA analysis of our samples, 96-well plates from the kit were first preincubated at 37°C for 30 minutes. Meanwhile, 50 μL standard, 50 μL quality control, and 50 μL plasma samples were diluted with the assay diluents and then loaded into 96-well microplates. Anti–Hsp90α-HPR-conjugated antibodies (50 μL) were added to the plates for incubation at 37°C for 1 hour. Then, the plates were washed four times with the washing buffer, and the reaction was visualized by adding 50 μL 3, 3, 5, 5-tetramethylbenzidine (TMB) substrate and incubated for 20 minutes at 37°C before stoppage with an acid stop buffer. Finally, optical density was measured using a spectrophotometer at 450 nm (the 620 nm was used as a reference wavelength). The amount of protein in each sample was calculated according to a standard curve of optical density values.

Detection of other tumor markers
The plasma levels of carcinoembryonic antigen (CEA) and CYFRA21-1 were also measured using a commercially available ELISA kit (R&D Systems), according to the manufacturer’s protocols.

Statistical analysis
Statistical analyses were performed using SAS9.2 software (SAS Institute Inc.). The data were summarized as mean. Differences (if any) in the first cohort data were evaluated using the t test or the rank-sum test. The differences in the second cohort data were evaluated using the Student’s t test. To assess the diagnostic performance of Hsp90α and Hsp90α in combination with CEA and CYFRA21-1 between patients with lung cancer and non–lung cancer, the models were assessed by logistic regression. Receiver operating characteristic (ROC) curves were generated to compare the predictive sensitivity, specificity, and the area under the curve (AUC) with 95% CI. The cutoff value for cancer diagnosis was determined by the Youden index. However, taking multiplicity controls into consideration, a P value less than 0.007 was considered statistically significant.

Results
In this study, we recruited a total of 2,247 participants (1,294 patients with lung cancer, 592 healthy controls, 344 patients with noncancerous lung lesions, and 17 patients with benign lung tumor). The lung cancer samples were divided into a static group of 1,046 patients and a dynamic group of 248 patients. The clinicopathologic characteristics from patients with lung cancer were obtained and are summarized in Table 1.

Differential plasma levels of Hsp90α protein in static group
The plasma levels of Hsp90α protein were assessed in the first cohort of 1999 participants using ELISA (Table 2 and Fig. 1). The data showed that levels of plasma Hsp90α protein were significantly higher in patients with lung cancer than those of the control groups (220.46 ng/mL vs. 48.00 ng/mL; P < 0.0001). The levels of plasma Hsp90α protein were higher in patients with lung cancer than those of the healthy controls (220.46 ng/mL vs. 40.32 ng/mL; P < 0.0001). Similarly, Hsp90α levels were higher in patients with lung cancer than in patients with noncancerous lung lesions (220.46 ng/mL vs. 58.58 ng/mL; P < 0.0001), while

| Table 1. Clinicopathologic characteristics of patients with lung cancer |
|-----------------------------|-----------------------------|-----------------------------|
| Characteristic              | Static group (n = 1,046)    | Dynamic group (n = 248)     |
|                             | Surgery (n = 79)            | Chemotherapy (n = 169)      |
| Sex                         | Male                        | 711                         | 48                          | 113                         |
|                            | Female                      | 331                         | 31                          | 55                          |
|                            | NA                          | 4                           | 1                           | 1                           |
| Stage                       | I                           | 65                          | 11                          | 0                           |
|                            | II                          | 28                          | 27                          | 0                           |
|                            | III                         | 214                         | 16                          | 24                          |
|                            | IV                          | 506                         | 1                           | 134                         |
|                            | NA                          | 233                         | 24                          | 11                          |
| Histologic type NSCLC       | Adenocarcinoma              | 537                         | 39                          | 101                         |
|                            | Squamous cell carcinoma     | 218                         | 16                          | 46                          |
|                            | Large-cell carcinoma        | 4                           | 2                           | 1                           |
|                            | SCLC                        | 136                         | 2                           | 11                          |
|                            | NA                          | 151                         | 20                          | 10                          |

Abbreviation: NA, not available.
Hsp90α was higher in patients with noncancerous lung lesions than in the healthy control group (58.58 ng/mL vs. 40.32 ng/mL; \( P < 0.0001 \)). However, there was no significant difference between patients with non–small cell lung cancer (NSCLC; 755 cases) and small cell lung cancer (SCLC; 136 cases) and between patients with adenocarcinoma (537 cases) and squamous cell carcinoma (218 cases).

We then determined ROC values and found that plasma Hsp90α protein levels >56.33 ng/mL would predict lung cancer cases with 72.18% (95% CI, 0.695–0.749) sensitivity and 78.70% (95% CI, 0.761–0.813) specificity. The AUC was 0.82 (Fig. 2).

### Table 2. The mean plasma levels of Hsp90α protein in the static group

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Hsp90α (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease group (lung cancer)</td>
<td>1,046</td>
<td>220.46</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I–II</td>
<td>93</td>
<td>111.50</td>
</tr>
<tr>
<td>III–IV</td>
<td>720</td>
<td>251.38</td>
</tr>
<tr>
<td>NA</td>
<td>233</td>
<td>139.83</td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>537</td>
<td>197.69</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>218</td>
<td>226.31</td>
</tr>
<tr>
<td>Large-cell carcinoma</td>
<td>4</td>
<td>407.23</td>
</tr>
<tr>
<td>SCLC</td>
<td>136</td>
<td>251.29</td>
</tr>
<tr>
<td>N/A</td>
<td>151</td>
<td>260.26</td>
</tr>
<tr>
<td>Control group</td>
<td>953</td>
<td>48.00</td>
</tr>
<tr>
<td>Healthy control</td>
<td>592</td>
<td>40.32</td>
</tr>
<tr>
<td>Noncancerous lung lesions</td>
<td>344</td>
<td>58.58</td>
</tr>
<tr>
<td>Benign lung tumor</td>
<td>17</td>
<td>106.98</td>
</tr>
</tbody>
</table>

Hsp90α was most sensitive for the diagnosis of lung cancer but displayed a lower specificity than CEA and CYFRA21-1.

Meanwhile, when we combined Hsp90α with CEA, the sensitivity reached 84.57% and the specificity was 74.93%. When we combined Hsp90α with CYFRA21-1, the sensitivity reached 86.97% and the specificity was 75.20%. Combining the markers could improve diagnostic sensitivity.
before chemotherapy and after two cycles of chemotherapy. Plasma levels of Hsp90α protein were higher after treatment in the PD group (367.67 ng/mL vs. 216.07 ng/mL, \( P < 0.0001 \)), whereas plasma levels of Hsp90α protein were lower after treatment in the PR and SD groups (178.90 ng/mL vs. 201.55 ng/mL, \( P < 0.0001 \)). We also assessed the change in tumor size after chemotherapy and found that the level of Hsp90α protein strongly correlated with tumor size (378.83 ng/mL vs. 227.23 ng/mL, \( P < 0.0001 \)).

**Discussion**

Lung cancer accounts for more than 25% of cancer-related deaths and about 15% of new cancer cases annually in the world (1). The identification of new tumor biomarkers could be pivotal in improvement of patient diagnosis and survival. In this study, we detected plasma levels of Hsp90 protein in a large cohort of patients with lung cancer, patients with noncancerous lung lesions, benign lung diseases, and healthy controls to assess its diagnostic value in lung cancer and treatment responses of patients with lung cancer. We found that plasma levels of Hsp90α protein were significantly higher in patients with lung cancer than in controls, and also higher in patients with advanced lung cancer (stage III-IV) than in patients with early-stage lung cancer (stage I–II). In contrast, there was no statistical difference between NSCLC and SCLC or between adenocarcinoma and squamous cell carcinoma. Using an optimum cutoff value, the levels of Hsp90α protein had a high sensitivity and specificity in diagnosis of lung cancer. Thus, the current study demonstrated that plasma Hsp90α protein levels are useful as a biomarker to detect lung cancer and predict the responses of patients with lung cancer to chemotherapy.

Indeed, Hsp90α protein is widely recognized as a chaperone and master regulator for the key cell signaling networks or cellular transcription in human cells. Recent studies have shown that secreted Hsp90α could activate matrix metalloproteinase-2 (MMP2) and thus facilitate maturation of MMP2 for promotion of tumor invasion (11–13). MMP2 is involved in the breakdown of extracellular matrix in normal physiological processes, and altered MMP2 expression contributes to human disease, including cancer development and progression. Another study showed a potentially common peptide target in secreted Hsp90α for hypoxia-inducible factor-1α–positive cancers (14). The authors localized the tumor-promoting effect of Hsp90α to a 115–amino acid region of secreted Hsp90α protein, maintenance of which was sufficient to bypass the blockade of HIF1α depletion and resumed tumor invasion,
sustaining that drugs that target these amino acid region could be more effective and less toxic in the treatment of HIF1α-positive cancers (14). Thus, altered expression of Hsp90α protein could serve as a biomarker for different human cancers.

In lung cancer, Wang and colleagues (10) demonstrated that plasma levels of Hsp90α protein were higher in most patients with cancer and was associated with cancer development, which could be a potential diagnostic and prognostic marker in clinical practice. A recent study showed that inhibition of Hsp90α reduced c-FLIPL level and, thus, c-FLIP and Hsp90α may be promising combined drug targets in human lung cancer treatment (15). More importantly, this protein may also have potential as a cancer-specific plasma biomarker for various human cancers, including lung cancer (16).Indeed, Hsp90α protein was one of 12 proteins used as a panel for lung cancer diagnosis, which were generated by a new aptamer-based proteomic analysis of 1,326 archived serum samples from four independent studies of NSCLC in long-term tobacco-exposed populations (16). Other previous studies showed the HSP90 inhibitor had a potent antitumor activity in in vitro and in vivo models of therapy against NSCLC (17, 18). Our current study further demonstrates that detection of plasma Hsp90α protein could be useful as a biomarker for diagnosis of lung cancer and for therapeutic monitoring of responses of patients with lung cancer. However, presently, a single protein used as a biomarker has a limitation for both sensitivity and specificity; thus, we, like others (16, 19, 20), combined different plasma proteins, to reach higher sensitivity and specificity.

The data from the current study showed that the combination of Hsp90α protein with CEA reached a sensitivity of 84.57%, while the specificity was 74.93% in diagnosis of lung cancer. When Hsp90α was combined with CYFRA21-1, the sensitivity reached 86.97% and the specificity was 74.93% in diagnosis of lung cancer (16). Indeed, Hsp90α protein was one of 12 proteins used as a panel for lung cancer diagnosis, which were generated by a new aptamer-based proteomic analysis of 1,326 archived serum samples from four independent studies of NSCLC in long-term tobacco-exposed populations (16). Other previous studies showed the HSP90 inhibitor had a potent antitumor activity in in vitro and in vivo models of therapy against NSCLC (17, 18). Our current study further demonstrates that detection of plasma Hsp90α protein could be useful as a biomarker for diagnosis of lung cancer and for therapeutic monitoring of responses of patients with lung cancer. However, presently, a single protein used as a biomarker has a limitation for both sensitivity and specificity; thus, we, like others (16, 19, 20), combined different plasma proteins, to reach higher sensitivity and specificity.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.K. Shi, X.H. Han
Writing, review, and/or revision of the manuscript (i.e., reporting or organizing data, constructing databases): Yantai Proten Biotechnology Development Co., Ltd
Study supervision: Y.K. Shi

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References


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