Development of Autoantibody Signatures as Novel Diagnostic Biomarkers of Non-Small Cell Lung Cancer

Running title: New serum diagnostic biomarkers for NSCLC

Lingling Wu¹, Wenjun Chang¹, Jinfeng Zhao¹, Yongwei Yu², Xiaojie Tan¹, Tong Su¹, Lijun Zhao³, Shengdong Huang⁴, Shiyuan Liu⁵ and Guangwen Cao¹

Authors’ Affiliations: ¹Department of Epidemiology, Second Military Medical University, Shanghai, China; ²Department of Pathology, The 1st Affiliated Hospital, Second Military Medical University, Shanghai, China; ³Department of Respiratory Diseases, The 1st Affiliated Hospital, Second Military Medical University, Shanghai, China; ⁴Department of Cardiothoracic Surgery, The 1st Affiliated Hospital, Second Military Medical University, Shanghai, China; ⁵Department of Diagnostic Imaging, The 2nd Affiliated Hospital, Shanghai, China

Grant support: This work was supported by Key Project of Shanghai Science and Technology Committee (06DZ19503 to GC) and Natural Science Foundation of China (30921006 to GC).

Note: L Wu and W Chang contributed equally to this work.

Institutions and Departments where the work was accomplished: Department of Epidemiology, Second Military Medical University, Shanghai, China.

Supplementary data for this article are available at Clinical Cancer Research
Online (http://clincancerres.aacrjournals.org/).

The sequence data described in this paper have been submitted to the NCBI GenBank under accession no. GU320337-GU320349.

Requests for reprints: Guangwen Cao, Department of Epidemiology, Second Military Medical University, 800 Xiangyin Rd., Shanghai, 200433, People’s Republic of China. Tel and fax: 86-21-81871060; E-mail: gcao@smmu.edu.cn.

Word count: Abstract, 250; text, 3743 (Statement of translational relevance, abstract, acknowledgement, references, legend and tables were not included); table, 3; figure, 3; and supplementary table, 7.
Statement of translational relevance

The study provided a 6-phage-peptide detector which had >92% sensitivity and >92% specificity in discriminating groups between non-small cell lung cancer (NSCLC) patients and healthy controls. The 6-phage-peptide detector could be used to diagnose NSCLC at early stage and discriminate NSCLC and chronic obstructive pulmonary diseases. Chemotherapy significantly reduced the seroreactivity of the 6 phage-peptide clones. The 6-phage-peptide detector can be used as a novel method for noninvasive diagnosis and screening of NSCLC in high risk populations. However, it is unlikely to be used as biomarkers for indicating the relapse of NSCLC after chemotherapy. From the 6 phage-peptide clones, we identified a new peptide target, olfactomedin 1, which was highly expressed in lung adenocarcinoma. This protein could be used as a potential target for the diagnosis and treatment of lung adenocarcinoma.
Abstract  

**Purpose:** To select autoantibody signatures as noninvasive biomarkers of non-small cell lung cancer (NSCLC).

**Experimental Design:** A phage cDNA expression library was constructed with fresh samples from 30 lung cancer patients and biopanned using serum pools of 10 NSCLC patients and 10 healthy controls. A 6-phage-peptide detector was discovered by two-step immunoscreenings, and validated in an independent set of 90 NSCLC patients and 90 matched healthy controls, 30 NSCLC patients with chemotherapy, and 12 chronic obstructive pulmonary disease (COPD) patients. Expression of a peptide target was validated by using immunohistochemistry. Factors affecting NSCLC-related death were evaluated by Cox regression analysis.

**Results:** Six phage-peptide clones showing higher seroreactivity than others in 30 NSCLC patients were selected for diagnostic validation. The 6-phage-peptide detector was able to discriminate between NSCLC patients and healthy controls with a sensitivity and specificity of greater than 92%, and had similar validity for indicating NSCLC at early stage. The seroreactivity of the 6 phage-peptides was significantly higher in the NSCLC patients than in those with chemotherapy and the COPD patients, respectively. Of the 6 phage-peptides, one encoded a peptide showing 100% homology...
to olfactomedin 1. Expression of olfactomedin 1 protein was significantly higher in lung adenocarcinoma than in lung cancer of other histological types and normal lung tissues. The autoantibody signature was not associated with the prognosis of the NSCLC patients.

**Conclusions:** The 6-phage-peptide-detector stands out as promising diagnostic biomarkers for NSCLC, unlikely for NSCLC relapse after chemotherapy. Olfactomedin 1 may be a novel target of lung adenocarcinoma.
Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer and one of the most common causes of cancer deaths worldwide (1). There are 1.2 million people in the world receiving a lung cancer diagnosis each year, only 16% of the cases are detected while the disease is still localized and possibly curable (2). Surgical resection is the most effective treatment for NSCLC without distant metastasis. The patients are usually diagnosed at late stage and lose the opportunity to be surgically excised. The patients diagnosed at early stage have a higher 5-year survival rate (49.5%) compared with those diagnosed at late stage (2.8%) (1). Early detection and diagnosis of NSCLC are of utmost importance in the therapy. Although recent advances in genomics and proteomics have generated many candidate tumor proteins in lung cancer samples (3-5), a blood test for NSCLC does not exist. The serum markers developed so far have not been recognized as ideal biomarkers due to the limited sensitivity and specificity. Most of these candidates, like CEA, neuron-specific enolase, chromogranine, CA125, and CA19-9, show an increased rate of positivity as the stage advances (6), and hardly serve as early biomarkers.

It has been proved that the immune system produces an antibody response to neoplastic cells, and B cells and their associated antibodies promote de novo carcinogenesis, suggesting that humoral immune responses may play a role in cancer progression (7). Patients with cancer...
usually produce detectable autoantibodies to certain tumor-associated antigens. Such autoantibodies, examined by using phage display, serologic analysis of recombinant cDNA expression libraries of human tumors with autologous serum, protein microarray, and serological proteome analysis, show increasing promise as diagnostic biomarkers for cancer patients (8-15).

Harnessing the immune response to tumor antigens is particularly useful for early detection of lung cancer because the immune response occurs early during tumor development (16). Autoantibodies to some known tumor-associated antigens have been found in the patients with lung cancer at the presymptomatic stage or before radiographic detection (16-19). Profiling cancer antigen–related autoantibodies has shown potential in early detection of lung cancer.

In this study, we described an approach to detect serum autoantibodies to NSCLC antigens, based on phage display technique and enzyme-linked immunosorbent assay (ELISA). We established a 6-phage-peptide detector which could be used to diagnose NSCLC at early stage and discriminate groups between the patients with NSCLC and the patients with chronic obstructive pulmonary diseases (COPD). This study also revealed that chemotherapy significantly reduced the immunoreactivity of circulating autoantibodies in the NSCLC patients to the 6 phage-peptides. These findings are useful for early diagnosis and monitoring of NSCLC.
Materials and Methods

**Study subjects and samples.** Sera of 130 patients with NSCLC before receiving any treatment, 30 NSCLC patients after receiving chemotherapy, 12 patients with COPD, and 100 healthy controls were collected at Department of Respiratory Diseases and Physical Examination Center, the 1st affiliated hospital of this university in August 2007-July 2009. The patients with other concurrent malignant diseases and autoimmune diseases were excluded. Serum from 5ml fasting blood was separated by centrifugation at 4°C and stored in a sterile tube at -80°C within 4h of sample collection. Clinical characteristics of the participants whose sera were used for immunoscreening and validation of the phage-peptide clones are presented in Table S1. Fresh primary lung cancer tissues were harvested from 30 patients who had not received chemotherapy and/or radiotherapy before surgical treatment at Department of Cardiothoracic Surgery, the 1st affiliated hospital of this university in March 2007 to May 2009 and frozen immediately in liquid nitrogen after surgical resection until used for cDNA phage library construction. Formalin fixed and paraffin embedded tumor specimens from 143 patients with lung cancer (77 patients with adenocarcinoma, 40 patients with squamous cell carcinoma, and 26 patients with small cell lung cancer) were from Department of Pathology of the 1st affiliated hospital and used for immunohistochemical analysis. Sera, fresh tumor samples, and formalin-fixed tumor specimens were from different participants. The study protocol conformed to the ethical regulations...
guideline of the 1975 Declaration of Helsinki, and was approved by the Institutional Review Board of this university. All participants who provided sera and those who provided fresh tumor tissues gave informed consents.

**RNA extraction and construction of T7 phage display cDNA expression library.** Total RNA was extracted separately according to a standard Trizol protocol. Message RNA was extracted by using PolyAT Tract mRNA Isolation System III (Promega, Madison, WI). Equal amounts of mRNA from fresh tumor samples of the 30 patients were pooled and reversely transcribed to cDNA with random primers, ligated with EcoRI/HindIII linkers, and cloned into T7 select 10-3b vectors arms of phage in T7Select® system (Novagen, Madison, WI) according to the manufacturer instructions. After *in vitro* packaging, a phage display library was obtained. The library was titered and amplified as previously described (8).

**Enrichment and screening of phage-peptide clones.** To enrich phage clones that bind specifically to autoantibodies associated with NSCLC, we performed 5 cycles of positive and negative selections with pooled sera of the 10 patients with NSCLC and the 10 healthy donors, as previously described (8). The phage library from the fifth cycle of biopannings was 1:10^8 diluted and allowed to grow on LB agar plates with carbenicillin (Sangon, Shanghai, China). Independent clones were randomly selected and identified with PCR amplification. The clones with inserts were further screened using ELISA with the pooled sera. PCR and ELISA were performed using T7Select® system.
(Novagen, Madison, WI) according to the manufacture’s instruction. The serum samples were 1:500 diluted, and used for ELISA. Blank controls (without serum sample), negative controls (with empty phages) and test wells of each selected phage were set in the same plate in triplicates. Normalized reactivity of each selected phage-peptide clone to the patients’ sera or the controls’ sera was calculated as followed: normalized reactivity = (average optical density [OD] value of the test wells – average OD value of the blank controls) / (average OD value of the negative controls – average OD value of the blank controls). PCR amplicons from high immunoreactive phage-peptide clones were subjected for direct DNA sequencing. After excluding the clones containing the same inserts, we examined the seroreactivity of each clone with individual serum samples harvested from another 30 NSCLC patients before receiving any treatment by using the same ELISA method. The clones with higher seroreactivity than others were then used to predict the probability of NSCLC in an independent set of 180 cases (90 patients with NSCLC and 90 age-, sex-matched healthy controls). Study flow for discovery and validation of the phage-peptide clones is shown in Fig.1.

**Nucleotide sequencing and bioinformatics analysis.** Direct DNA sequencing of the amplicons with T7 primers was carried out by using ABI PRISM BigDye sequencing kits and an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Phage identity was identified based on nucleotide sequence and deduced amino acid sequence matched with a single
molecule in GenBank database using nucleotide BLAST and protein BLAST search engines. The epitopes of these polypeptides according to Jameson-Wolf antigenicity index were predicted by DNAstar software (http://www.dnastar.com/). The sequences of the epitopes were again analyzed by protein blast engine for homology. A known protein showing great homology to an exogenous peptide of the phage-peptide clones was selected for immunohistochemical analysis.

**Immunohistochemistry.** Rabbit polyclonal antibody to human olfactomedin 1 (OLFM1) (Abgent, San Diego, USA) was used for immunohistochemical analysis according to a protocol described previously (20). Briefly, the deparaffinised sections were incubated with 20% goat serum for 30min to block nonspecific binding, and then incubated with 1:50 diluted primary antibodies for 30min in room temperature. The immunostaining was performed using the EnVision method (DAKO, Glostrup, Denmark) according to the manufacturer’s instructions. The images were obtained with a BX50 microscope (Olympus, Tokyo, Japan) equipped with a digital color video camera. All of the samples were analyzed by three independent observers (Y.Y. W.C, and L.W) who were blinded to the clinical information. Scoring of intensity of the immunostaining was done semiquantitatively (1+ = weak staining, 2+ = moderate staining, and 3+ = strong staining). The cases were interpreted as negative if <10% of the cells were positive. There was a close agreement (>90%) among the investigators. Disagreements were resolved by consensus.
Follow-up of the patients involved in the validation study. A total of 90 NSCLC patients whose sera were used for the validation of the autoantibody signature were followed up. All data, including sex, age, chemotherapy, radiotherapy, surgical treatment, and histopathologic data, such as tumor type and stage, were obtained from the clinical and pathologic records. Follow-up was performed by examining them every 3 months on outpatient bases and/or by phone calls.

Statistical analysis. To determine whether or not the autoantibody signature could be used for the noninvasive detection of NSCLC, independent samples t test was used to compare the normalized reactivity of circulating autoantibodies between NSCLC patients and healthy controls. For the evaluation of autoantibodies to the selected phage clones in combination as an indicator of NSCLC, logistic regression, principal component regression, and support vector machine were used for the establishment of classifiers, respectively. The discriminatory capacity of individual or combination of the selected phage clones was also evaluated by the receiver operating characteristic curve. The area under the curve (AUC) and their 95% confidence interval (CI) were calculated. The classifiers were then examined by using leave-one-out cross-validation (LOOCV) within all the serum samples. Wilcoxon rank sum test was used to evaluate positive degrees of the immunostaining for OLFM1 in lung adenocarcinoma, squamous cell carcinoma, small cell lung carcinoma, and pathologically normal lung tissues. Cox
regression models allowed for univariate and multivariate analyses of survival risk factors, including age, sex, tumor stage, treatment after harvesting sera, and the normalized reactivity of autoantibodies to each of the 6 phage-peptide clones. All statistical tests were two-sided and done with digeR package (21) or Statistical Program for Social Sciences (SPSS 15.0 for Windows, SPSS, Chicago, IL). \( P < 0.05 \) was considered as statistically significant.

**Results**

**Screening and identification of phage-peptide clones exhibiting high reactivity with NSCLC sera.** With the use of mRNA of tumor samples of the 30 patients with histologically confirmed lung cancer, we constructed a phage-display library of lung cancer containing \( 1.8 \times 10^6 \) primary recombinants. After 5 rounds of the biopannings, we randomly picked up 2,000 phage clones for PCR identification. A total of 1,212 of the 2,000 clones contained exogenous DNA inserts. After ELISA screening of the 1,212 clones with the serum pools, we selected 22 clones that had high reactivity with NSCLC pooled sera (normalized reactivity > 2.0) but low reactivity with healthy control pooled sera (normalized reactivity < 1.5) for direct DNA sequencing of the inserts. Table S2 shows the deduced amino acid sequences and epitope sequences of the 22 inserts. Of the 22 clones, 14 contained independent inserts. Parts of the 14 translated polypeptides showed 62%-100% homology to known human proteins, and 2 (no. 96 and no. 252) showed no homology to...
any known protein. Three polypeptides (no. 72, no. 290, and no. 357) and the epitopes were in-frame and within known sequences of major histocompatibility complex, class I, B (HLA-B), OLFM1, and ribosomal protein L23a (RPL23A), respectively. A peptide encoding OLFM1, spanning 64 amino acids (aa) from aa33 to aa96, was found in 3 independent phage-peptide clones. DNA sequences of 13 of the 14 independent inserts, excluding clone no.252 with an insert less than 50bp, were submitted to GenBank with accession no. GU320337-GU320349. The 14 independent clones were further screened by ELISA with individual serum samples of the 30 NSCLC patients. The first 6 phage clones (no.72, no.91, no.96, no.252, no.286, and no.290) with higher seroreactivity than others were selected as candidate NSCLC biomarkers.

**Autoantibody signature for the diagnosis of NSCLC.** We next determined whether the autoantibody signature could be used as an indicator of NSCLC. The 6 phage-peptide clones were subjected to serologic analysis in an independent set of 180 cases (90 cases of NSCLC patients and 90 cases of age-, sex-matched healthy controls). Each of the 6 clones showed a significant difference between cases and controls ($P < 0.001$ for each clone) (Fig.2A). A logistic regression method with LOOCV was initially used to determine whether or not one of the selected clones exhibited differential reactivity with each serum sample of the 90 NSCLC cases relative to the 90 healthy controls. Autoantibodies to each of the 6 phage peptides were able to discriminate
between the NSCLC patients and healthy controls significantly, with an AUC of 0.810 to 0.908 (Fig.2B). Autoantibodies to the phage-peptide clone encoding OLFM1 exhibited an AUC of 0.810. A logistic regression model with LOOCV revealed that the sensitivity and specificity of a panel of the 6 phage-peptide clones were 92.2% and 92.2%, respectively. Similar accuracy was achieved by using principle component regression and support vector machine models, as shown in Table 1. The seroreactivity of each of the 6 phage-peptide clones did not differ between adenocarcinoma and squamous cell carcinoma ($P > 0.05$ for each).

To evaluate the ability of the detector for the discrimination of NSCLC patients at early stage and healthy controls, sera of 21 NSCLC patients at I and II stages and the 90 healthy controls were used for the validation. With the use of a logistic regression model with LOOCV, the ability of the panel of 6 phage-peptides to discriminate between the patients at early stage and the healthy controls was significant ($P < 0.001$), with an AUC of 0.888 (95% CI, 0.781-0.996). Moreover, the most high prediction accuracy was obtained by using support vector machine with LOOCV, with an AUC of 0.962 (95% CI, 0.923-1.001), as shown in Table 1. Thus, the 6-phage-peptide detector had greater than 92% sensitivity and greater than 85% specificity for NSCLC at early stage.

**Effect of chemotherapy on the immunoreactivity.** To evaluate the effect
of chemotherapy on the seroreactivity of the 6 phage-peptide clones, we compared the normalized reactivity of autoantibodies against each of the 6 phage-peptide clones among 30 NSCLC patients who had received chemotherapy (Table S3), 30 age-, sex-matched NSCLC patients without chemotherapy, and 30 age-, sex-matched healthy controls. The seroreactivity of each of the six clones was significantly lower in the patients with chemotherapy than in those without chemotherapy ($P < 0.01$ for each), as shown in Table 2.

The seroreactivity of the 6 phage-peptide clones in the patients with COPD. We examined the normalized reactivity of autoantibodies in 12 patients with COPD, the 90 patients with NSCLC and the 90 healthy controls to the 6 phage-peptide clones. The normalized reactivity of autoantibodies to each of the 6 clones did not have statistical differences between the COPD patients and healthy controls, while the differences were significant between the NSCLC patients and the COPD patients (Table 3).

Expression of OLFM1 protein in lung cancer. Expression pattern of OLFM1 protein in 52 pathologically normal lung tissues, 77 lung adenocarcinoma, 40 lung squamous cell carcinoma, and 26 small cell lung cancer specimens was examined using immunohistochemistry. Table S4 shows the characteristics of the 143 patients whose specimens were used in immunohistochemistry. OLFM1 staining was predominantly cytoplasmic in all
positive cells (Fig.3). Wilcoxon rank sum test demonstrated that OLFM1 protein was significantly up-regulated in lung adenocarcinoma ($P < 0.01$) and lung squamous cell carcinoma ($P < 0.01$) than in the pathologically normal lung tissues, respectively. Furthermore, OLFM1 antigen was significantly up-regulated in adenocarcinoma than in squamous cell carcinoma ($P < 0.01$) (Table S5). Expression of OLFM1 antigen was not significantly different between adenocarcinoma at early stage (I+II) and adenocarcinoma at late stage (III+IV) ($P = 0.972$) (Table S6).

**Association of the autoantibody signature with the prognosis.** Of the 90 NSCLC patients, 6 failed to be successfully followed up. The 84 cases (55 males, 29 females) were involved in the survival analysis. After a median observation time of 17.8 months (ranging from 0.3 to 82.2 months), 32 patients died of NSCLC. Univariate Cox regression analysis showed that TNM stage (hazard ratio [HR], 2.22; 95% CI, 1.28-3.86) and surgical treatment (HR, 0.23; 95% CI, 0.10-0.54) were significantly associated with cancer-related death, respectively. However, the normalized reactivity of autoantibodies to each of the 6 phage-peptides was not associated with cancer-related death (Table S7). Multivariate Cox regression analysis showed that surgical treatment was solely associated with cancer-related death (adjusted HR, 0.23; 95% CI, 0.10-0.54).

**Discussion**

In this study, we took 3 steps in the discovery phase to select the
phage-peptide clones. First, NSCLC-specific phage-peptide clones were enriched by using biopannings. Second, 22 clones that had high reactivity with NSCLC pooled sera but low reactivity with healthy control pooled sera were selected for the identification of the peptide targets. Third, the first 6 phage clones with higher immunoreactivity than others were selected by using individual serum samples of another 30 NSCLC patients. This strategy enables discovery of phage-peptide clones with high sensitivities and specificities for NSCLC. We also used ELISA screenings to replace the phage-peptide microarray step (8). This change decreased the complexity of screening procedure and enabled discovery of the 6-phage-peptide detector without the need for high-precision instruments. To minimize background signals of ELISA, the seroreactivity of each phage-peptide was measured in relation to an internal control signal detected by antibody against phage capsid proteins. Analyses of repeated experiments using same sera showed a quite consistent result of ELISA. Compared with other reports for the identification of tumor biomarkers by using phage display (8, 9, 11, 13-15, 17, 18), this approach is relatively simple and cost-effective.

With the use of a logistic regression and LOOCV, we found that each of the 6 phage-peptides was able to discriminate NSCLC samples and healthy control samples significantly, with an AUC of 0.810 to 0.908. By using the same model, the 6-phage-peptide detector yielded an AUC of 0.956 in NSCLC. Thus, the 6-phage-peptide detector is more reliable for the diagnosis
of NSCLC than each of the 6 phage-peptide clones. Since NSCLC is a heterogeneous disease, a panel of autoantibodies that covers the broad clinical phenotype is needed (2). To validate if the 6-phage-peptide detector was suitable for early diagnosis of NSCLC, we used sera of 21 NSCLC patients at I and II stages and 90 healthy controls for validation. The 6-phage-peptide detector yielded an AUC of 0.962 by using support vector machine model with LOOCV and has equal ability to diagnose early NSCLC and all involved NSCLC, indicating that it has the potential value for early diagnosis and screening of NSCLC in high-risk populations.

An important finding of this study was that the seroreactivity of each of the 6 phage-peptide clones was significantly lower in the patients with chemotherapy than in those without chemotherapy. Chemotherapy significantly reduced the levels of autoantibodies against the 6 phage-peptide clones, probably due to the inhibitory role of chemotherapy on B cell-dependent humoral immunity. Thus, the 6 phage-peptide clones were unlikely to be used as biomarkers for indicating the recurrence of NSCLC after chemotherapy.

This study revealed that the seroreactivity of each of the 6 phage-peptide clones was significantly lower in the patients with COPD than in the patients with NSCLC. COPD is associated with autoimmunity, with activation of dendritic cells and T-helper cells of both type 1 and 2, and the senescence
response (22). Chronic inflammation is an important cause of COPD. The 6 phage-peptide clones did not react with autoantibodies in the COPD patients apparently, indicating that the autoantibodies to the 6 phage-peptides might be less associated with chronic inflammation. The 6-phage-peptide detector is able to discriminate between NSCLC and COPD although COPD has been established as an independent risk factor for lung cancer (23).

The levels of autoantibodies to given antigens have been shown to have prognostic value for lung cancer (24, 25). In this study, the levels of autoantibodies to the 6 phage-peptide clones were not statistically associated with cancer-related death of the NSCLC patients, indicating that the autoantibody signature for the diagnosis is unlikely to be used to predict the prognosis of NSCLC. This may be related to the strategy of screening the autoantibody signature. The autoantibody signature for the diagnosis of NSCLC was derived from sera of the patients relative to healthy controls, not from sera of the patients with different prognosis.

Of the 22 phage-peptides with their insets identified, clone no. 290 is the only phage-peptide that shows completely homology to a known human protein OLFM1. The 66aa OLFM1 peptide contains potential epitopes that could elicit immune responses. Cellular localization and expression of OLFM1 protein in lung cancer of various histological types and pathologically normal lung tissues were examined using immunohistochemistry. Strong cytoplasmic
staining of OLFM1 was observed in NSCLC, especially in adenocarcinoma, compared with the pathologically normal lung tissues. No difference in OLFM1 expression was found in adenocarcinoma at early stage and at late stage (Table S6), implying that this antigen could elicit a humoral immune response at early stage of lung adenocarcinoma. OLFM1 was significantly up-regulated in adenocarcinoma than in squamous cell carcinoma ($P < 0.01$), whereas the seroreactivity of phage clone no.290 did not differ between adenocarcinoma and squamous cell carcinoma, indicating that OLFM1 protein in squamous cell carcinoma is less likely to elicit a humoral immune response in situ. OLFM1, also called Noelin-1, is a secreted glycoprotein belonging to a family of olfactomedin domain-containing proteins and plays an important role in regulating the production of neural crest cells by the neural tube (26). OLFM1 is deregulated in the endometrial cancer, Ewing’s sarcoma, and neuroblastoma (27-29). The role of OLFM1 on the development of NSCLC is unclear and warrants further investigation.

Several limitations should be addressed. Only 12 patients with COPD were included in the validation study. Although the normalized reactivity of autoantibodies to the 6 phage-peptide clones was quite consistent among the COPD patients, more patients were needed to increase the reliability. CEA and other possible serum biomarkers were not used for evaluating the validity of the 6-phage-peptide detector. Also, we were unable to match smoking state between the NSCLC patients and healthy controls, resulting in loss of data.
In summary, this study suggests that a novel autoantibody signature may be useful for the screening and early diagnosis of NSCLC due to the >92% sensitivity and >85% specificity of the assay. Autoantibodies to the 6-phage-peptide detector are able to discriminate the NSCLC patients, the COPD patients, and healthy subjects, but might be unlikely to indicate the relapse of NSCLC after chemotherapy. Of candidate peptide targets detected in this study, OLFM1 is suggested to be a new target of NSCLC. The value of this technique for early diagnosis of NSCLC needs to be further evaluated by using sera from patients with various pathological conditions.
Acknowledgements

The authors thank Ms. Yan Liu (Department of Epidemiology, Second Military Medical University, Shanghai, China) for some technical assistance and Dr. Yaoliang Pan (Physical Examination Center, the 1st affiliated hospital, Second Military Medical University, Shanghai, China) for his help in recruiting healthy controls.
Reference


Figure Legends

Figure 1. Study flow for discovery and validation of the phage-peptide detector.

Figure 2. Diagnostic values of the 6 candidate phage-peptide clones in an independent set of 90 NSCLC patients and 90 healthy controls. A, the normalized reactivity of autoantibodies to the 6 phage-peptide clones. The box area contains 50% of the data samples, and 99.3% of the samples are within the upper and lower boundary markers. B, the receiver operating characteristics curves and AUC values of the 6 phage-peptide clones. Solid lines and dot lines indicate the statistical models with or not with leave-one-out cross-validation, respectively.

1. Library construction

- Fresh tumor samples from 30 patients with lung cancer
- mRNA pooled
- Reverse transcription
- T7 phage system
- Cloned into T7 select 10-3b

2. Immunoscreening and bioinformatics

- Biopanned with pooled sera from 10 NSCLC patients and 10 healthy controls
- 2000 phage clones were randomly selected
- 1212 recombinant clones
- 22 phage-peptides with normalized reactivity > 2.0 in NSCLC and normalized reactivity < 1.5 in controls
- DNA sequencing of the inserts
- BLASTn, BLASTp and epitope prediction
- ELISA screening with individual serum samples of 30 NSCLC patients

3. Validation

- Validated in an independent set of 90 NSCLC patients and 90 healthy controls
- Validity of a 6-phage-peptide detector for the diagnosis of NSCLC and NSCLC at early stage
- Effect of chemotherapy on immunoreactivity of autoantibodies to the 6-phage-peptides
- Immunoreactivity of autoantibodies in the patients with COPD to the 6-phage-peptides
- Immunohistochemical validation
A

Clone No.72

Normalized reactivity

(P<0.001)

Control NSCLC

Clone No.252

Normalized reactivity

(P<0.001)

Control NSCLC

Clone No.96

Normalized reactivity

(P<0.001)

Control NSCLC

B

Clone No.72

Sensitivity

AUC=0.912

AUC=0.905

Clone No.91

Sensitivity

AUC=0.902

AUC=0.897

Clone No.96

Sensitivity

AUC=0.913

AUC=0.908

Clone No.252

Sensitivity

AUC=0.894

AUC=0.887

Clone No.286

Sensitivity

AUC=0.912

AUC=0.887

Clone No.290

Sensitivity

AUC=0.818

AUC=0.810
Table 1. Validity of the autoantibody signature for the discrimination of the NSCLC patients and healthy controls by using three models with leave-one-out cross validation

<table>
<thead>
<tr>
<th>Model</th>
<th>AUC (95% CI)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For all stage (I-IV) NSCLC (NSCLC patients = 90, healthy controls = 90)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Logistic regression</td>
<td>0.956</td>
<td>92.2%</td>
<td>92.2%</td>
<td>92.2%</td>
<td>92.2%</td>
</tr>
<tr>
<td></td>
<td>(0.923-0.989)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Principal component regression</td>
<td>0.956</td>
<td>92.2%</td>
<td>92.2%</td>
<td>92.2%</td>
<td>92.2%</td>
</tr>
<tr>
<td></td>
<td>(0.924-0.989)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Support vector machine</td>
<td>0.969</td>
<td>95.6%</td>
<td>95.6%</td>
<td>95.6%</td>
<td>95.6%</td>
</tr>
<tr>
<td></td>
<td>(0.938-1.000)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>For early stage (I-II) NSCLC (NSCLC patients = 21, healthy controls = 90)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Logistic regression</td>
<td>0.888</td>
<td>92.2%</td>
<td>85.7%</td>
<td>59.4%</td>
<td>97.5%</td>
</tr>
<tr>
<td></td>
<td>(0.781-0.996)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Principal component regression</td>
<td>0.935</td>
<td>94.4%</td>
<td>90.5%</td>
<td>71.4%</td>
<td>98.8%</td>
</tr>
<tr>
<td></td>
<td>(0.867-1.004)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Support vector machine</td>
<td>0.962</td>
<td>96.7%</td>
<td>95.2%</td>
<td>83.3%</td>
<td>98.9%</td>
</tr>
<tr>
<td></td>
<td>(0.923-1.001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: NPV, negative predictive value; PPV, positive predictive value.
Table 2. The seroreactivity of the 6 phage-peptide clones in the sex-, age-matched NSCLC patients with or without chemotherapy and healthy controls

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Normalized reactivity (means±standard deviation)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NSCLC patients with chemotherapy (n=30)</td>
<td>NSCLC patients without chemotherapy (n=30)</td>
<td>Healthy controls (n=30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(means±standard deviation)</td>
<td>(means±standard deviation)</td>
<td>(means±standard deviation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.72</td>
<td>0.80±0.34</td>
<td>2.19±1.58</td>
<td>0.98±0.47</td>
<td>0.000</td>
<td>0.097</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.91</td>
<td>0.77±0.40</td>
<td>1.86±0.80</td>
<td>0.90±0.36</td>
<td>0.000</td>
<td>0.159</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.96</td>
<td>0.96±0.48</td>
<td>1.78±1.03</td>
<td>0.79±0.46</td>
<td>0.000</td>
<td>0.182</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.252</td>
<td>1.06±0.62</td>
<td>1.92±0.91</td>
<td>0.78±0.36</td>
<td>0.000</td>
<td>0.050</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.286</td>
<td>1.05±0.48</td>
<td>1.81±0.65</td>
<td>0.85±0.34</td>
<td>0.001</td>
<td>0.092</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No.290</td>
<td>0.92±0.49</td>
<td>1.81±1.08</td>
<td>0.78±0.38</td>
<td>0.000</td>
<td>0.202</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*NSCLC patients with chemotherapy vs. NSCLC patients without chemotherapy; †NSCLC patients with chemotherapy vs. healthy controls.
Table 3. The seroreactivity of the 6 phage-peptide clones in the COPD patients, NSCLC patients, and healthy controls

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>COPD patients (n=12)</th>
<th>NSCLC patients (n=90)</th>
<th>Healthy controls (n=90)</th>
<th>P-value*</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.72</td>
<td>0.80±0.22</td>
<td>2.20±1.37</td>
<td>1.01±0.48</td>
<td>0.001</td>
<td>0.148</td>
</tr>
<tr>
<td>No.91</td>
<td>0.83±0.14</td>
<td>2.10±1.03</td>
<td>0.96±0.46</td>
<td>0.001</td>
<td>0.332</td>
</tr>
<tr>
<td>No.96</td>
<td>0.84±0.24</td>
<td>2.13±1.10</td>
<td>0.89±0.50</td>
<td>0.000</td>
<td>0.732</td>
</tr>
<tr>
<td>No.252</td>
<td>0.82±0.23</td>
<td>2.04±1.35</td>
<td>0.88±0.41</td>
<td>0.003</td>
<td>0.655</td>
</tr>
<tr>
<td>No.286</td>
<td>0.90±0.21</td>
<td>1.94±0.89</td>
<td>0.92±0.41</td>
<td>0.000</td>
<td>0.932</td>
</tr>
<tr>
<td>No.290</td>
<td>0.92±0.25</td>
<td>1.93±1.39</td>
<td>0.91±0.45</td>
<td>0.014</td>
<td>0.953</td>
</tr>
</tbody>
</table>

* COPD patients vs. NSCLC patients; † COPD patients vs. healthy controls.
Development of Autoantibody Signatures as Novel Diagnostic Biomarkers of Non-Small Cell Lung Cancer

Ling-Ling Wu, Wen-Jun Chang, Jinfeng Zhao, et al.

Clin Cancer Res  Published OnlineFirst May 25, 2010.