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# Molecular Recognition of Small-Cell Lung Cancer Cells Using Aptamers

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*Early diagnosis is the way to improve the rate of lung cancer survival, but is almost impossible today due to the lack of molecular probes that recognize lung cancer cells sensitively and selectively. We developed a new aptamer approach for the recognition of specific small-cell lung cancer (SCLC) cell-surface molecular markers. Our approach relies on cell-based systematic evolution of ligands by exponential enrichment (cell-SELEX) to evolve aptamers for whole live cells that express a variety of surface markers representing molecular differences among cancer cells. When applied to different lung cancer cells including those from patient samples, these aptamers bind to SCLC cells with high affinity and specificity in various assay formats. When conjugated with magnetic and fluorescent nanoparticles, the aptamer nanoconjugates could effectively extract SCLC cells from mixed cell media for isolation, enrichment, and sensitive detection. These studies demonstrate the potential of the aptamer approach for early lung cancer detection.*

## Introduction

As the leading cause of cancer mortality, lung cancer accounts for 29% of all cancer deaths in the United States with a five-year survival rate of less than 15%.[1] The main reason for the high death rate of lung cancer patients is that they are diagnosed at an advanced stage, when treatments are rarely successful.[2,3] Among all the lung cancer subtypes, small-cell lung cancer (SCLC) has the highest tendency for early dissemination and the shortest median survival (7–12 months) as a clinically distinct entity.[4,5]

Survival of patients with lung cancer, especially SCLC, relies on early detection as well as effective treatment.[6] Although the previous clinical trial of early detection using sputum cytology and chest X-rays led to earlier diagnosis of lung cancers, no decrease in overall mortality could be observed thereafter.[7] The recent development of imaging-based screening technologies such as spiral computed tomography (CT), optical coherent tomography, positron emission tomography (PET), virtual bronchoscopy, autofluorescence bronchoscopy, and confocal microscopy has not shown to improve this situation either.[7,8] The possible reason for this is that morphological criteria used in imaging approaches are not sensitive enough during the pre-malignant phase of lung cancer development,[9,10] especially SCLC, which arises without morphologically recognizable pre-neoplastic lesions.[11]

To further improve early detection, molecular approaches were exploited to complement imaging studies. Molecular abnormalities correlate with behavioral aspects of lung cancer, and are therefore more sensitive in detecting invasive malignant lesions in preclinical phase than tumor sizes revealed by imaging. Genetic and proteomic analysis are two major molecular-marker-based early detection techniques.[12] Genetic changes can be detected reproducibly by PCR[13] and genomic hybridization,[14] but they do not always correlate with changes at the protein level. Proteomic analysis using immunostaining[15] and mass spectrometry[16] is a new trend of early detection. However, there has been only limited success in developing sensitive and specific molecular probes for such analysis.[17]

Herein we describe a new nucleic acid probe-based approach, in which a panel of DNA aptamers was purposely developed against the molecular differences among lung cancer cells to detect specific molecular markers on SCLC cell surfaces. Our approach relies on cell-SELEX (systematic evolution of ligands by exponential enrichment) to ensure the specificity and widespread availability of aptamer probes, which are missing in antibody-based methods.[18–20]

With cell-SELEX, we developed a panel of molecular aptamers for SCLC, which is the most aggressive lung cancer subtype, with greater than 95% mortality in a few years and which imparts the shortest life expectancy among all lung cancer subtypes.[21,22] These aptamer probes were selected without prior knowledge about SCLC biomarkers and tested for their ability to specifically bind both cultured cells and clinical samples of SCLC in various assay formats. Aptamers were also exploited for detection and enrichment of SCLC cells, a critical step towards the goal of early detection, when sensitive detection is needed.

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In comparison with other molecular recognition elements, aptamers used in this approach present several advantages for early detection.[23–25] While the aptamers' sensitivity leads to the detection of malignant cells, their specificity derived from cell-SELEX prevents cross-reactivity with normal epithelial cells, resulting in fewer false positives. In practice, multiple aptamers can be readily developed for any cancer cells of interest without prior knowledge of cell-surface marker proteins, and are more predictive of cancer progression than single probes used in previous studies. In addition, low-molecular-weight aptamers can be easily synthesized and modified to recognize the target proteins at their native state on cell surfaces reproducibly.

## Results and Discussion

### SELEX for whole live cancer cells

To develop cell-specific aptamer probes, live cancer cells were directly used as the target for cell-SELEX, an approach previously established in our research group. This approach was adapted in a few aspects to work with floating aggregates of SCLC and adherent monolayers of non-small-cell lung cancer (NSCLC), which are two typical growth patterns of lung cancer culture. Cell-SELEX with lung cancer cells is generally more difficult to do than with leukemia cells,[20,26] due to heterogeneity of cell population and poor viability. We chose to develop aptamer probes that target SCLC (classic) in an initial study as it possesses the worst clinical presentation among all lung cancer phenotypes. NSCLC (large cell) was adopted as a control for cell-SELEX to generate aptamers exclusive to the cell-surface markers of SCLC. These cell-surface markers are so exclusive to SCLC that normal lung epithelial cells are also not expected to bear them and cross-react with developed aptamers, as observed in previous studies with antibodies.[10] With counter-selection against control cells, the aptamers achieve the great selectivity necessary for reliable detection of lung cancer antigens.

In the selection (Figure 1 A), a cultured SCLC cell line, NCI-H69, was first incubated with a 71-base synthetic single-stranded DNA library. The DNA sequences that bound to target cells were then eluted after stringent washing. A cultured NSCLC cell line, NCI-H661, was then introduced as a control to separate aptamers with affinity for both the target and control cells from those aptamers that recognize only target cells in the previously eluted DNA pool. The remaining target-cell-specific sequences from counter-selection were further PCR amplified to form the starting pool of the next round of selection. A panel of aptamer probes eventually evolved to have great specificity and high affinity for SCLC along with the progression of SELEX cycles.

### Enrichment of aptamers

We monitored the gradual enrichment of aptamers during the selection process by both flow cytometry (Figure 1 B) and confocal microscopy (data not shown). The ability of DNA pools from each round of selection to bind target cells was assessed. The increase in fluorescence intensity of the dye-labeled DNA pools bound to target cells is gradual and steady along with the progress of selection, indicating a successful evolution of high-affinity aptamers. By contrast, no significant change was observed in the response to the control cells during the selection process, demonstrating the specificity of selected DNA pools.

### SCLC-specific aptamers

After 25 rounds of selection, the binding ability of the selected DNA pool reached a plateau, and cloning was performed to isolate individual aptamers in the most selected DNA pool. Results of subsequent sequencing were further analyzed by multiple sequence alignment software (Figure S1, Supporting Information). We found that the majority of aptamers in the selected pool belong to several families based on the consensus sequences they have.

To deconvolute the selected DNA pool, those consensus sequences with high repeats were synthesized to test their ability to specifically bind SCLC cells. A few of them showed prominent binding affinity toward SCLC but not NSCLC (control cells), as illustrated by the results of flow cytometry (Figure 2 A). The dominant peak refers to the binding of aptamer with SCLC cells. A second peak with high fluorescence signal was also noted, which may represent the population of dead cells. According to confocal imaging results, fluorescent dye-labeled aptamers only specifically bind to target SCLC cells (Figure 2 B). In addition, individual aptamers were tested with saturation analysis as depicted in Figure S2 and were found to have high affinity with equilibrium dissociation constants in the nanomolar range (Table 1).

**Table 1.** Equilibrium dissociation constants of selected SCLC aptamers.

| Selected sequence name | $K_d$ [nM] |
|---|---|
| HCA12 | ~97 |
| HCC03 | ~123 |
| HCH07 | ~38 |
| HCH01 | ~157 |

### Enzymatic treatment of cell-surface markers

In addition to the previous studies of developed aptamers, their putative cell-surface targets were examined by enzymatic treatment to further verify the binding of aptamers to SCLC cell-surface markers. After brief treatment of cells with trypsin or proteinase K, diminished binding of aptamers to SCLC cells was observed by flow cytometry in both cases (Figure S3 A). We observed the same trend under confocal microscopy; only a limited amount of fluorescent aptamers were retained on enzyme-treated cell surfaces (Figure S3 B). These results suggest that selected aptamers do indeed bind to cell membrane target molecules, and these discovered SCLC cell-surface markers can be affected by protease.

  

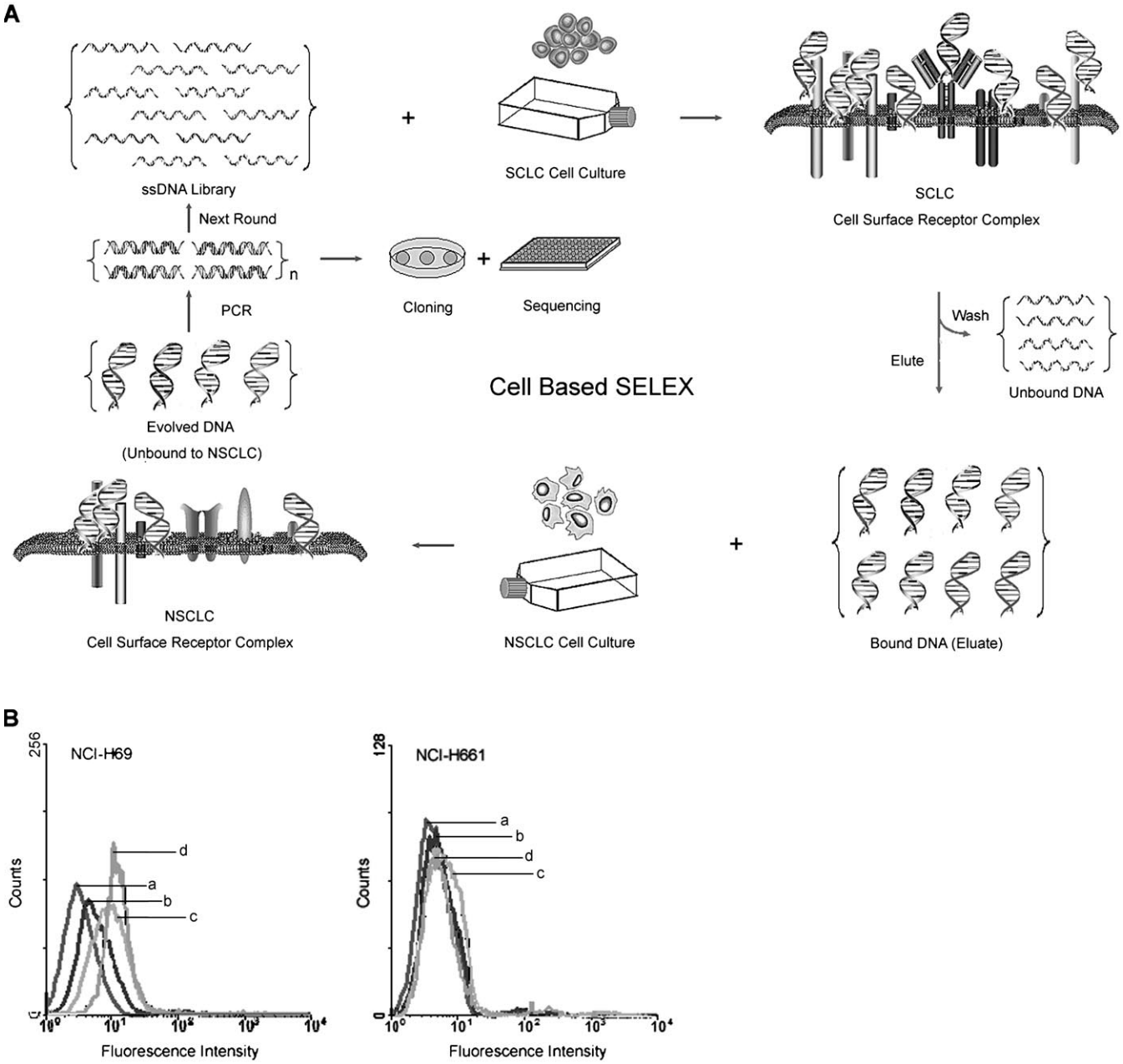


**Figure 1.** Process of cell-SELEX for SCLC and monitoring of the enrichment of aptamers along the progress of SELEX. A) A number of DNA molecules from the ssDNA library bind to SCLC cells after incubation and are retained for counter-selection with NSCLC cells. The SCLC-specific DNA molecules are subsequently PCR amplified for the next round of selection, or for cloning and sequencing to identify individual aptamers in the most selected pool. B) Gradual evolution of SCLC-specific aptamers along with the progress of SELEX. The FITC-labeled ssDNA library and selected DNA pools were tested for binding to NCI-H69 (SCLC, left) and NCI-H661 (NSCLC, right) cells by flow cytometry. The binding ability of selected DNA pools gradually increased for SCLC, and no significant change was observed for NSCLC: a) ssDNA library, b) 5th round pool, c) 18th round pool, d) 25th round pool.

### Validation of aptamers with various cancer cells and assay formats

Before testing with clinical samples, we assessed the applicability of developed aptamer probes to other cultured SCLC cell lines (that is, to validate the target molecules of developed aptamers as exclusive markers for SCLC). The panel of aptamers showed consistent binding patterns to NCI-H146 and NCI-H128 (Table 2), two SCLC cell lines that have similar cell characteristics as NCI-H69 (the target cell line used in cell-SELEX). In contrast to SCLC, three NSCLC cell lines, adenocarcinoma, squamous-cell carcinoma, and large-cell carcinoma (the control cell line used in cell-SELEX), did not respond to the selected aptamers except in one case (aptamer HCH07 bound to NCI-H23; Table 2). Moreover, other cancer types including two leukemia cell lines and two liver cancer cell lines were not recognized by these aptamers in most cases (Table 2). Interestingly, the aptamer that bound to adenocarcinoma NCI-H23 is also able to recognize liver cancer cell lines. Considering these cell lines were not used as control cells in cell-SELEX, there is still a

  

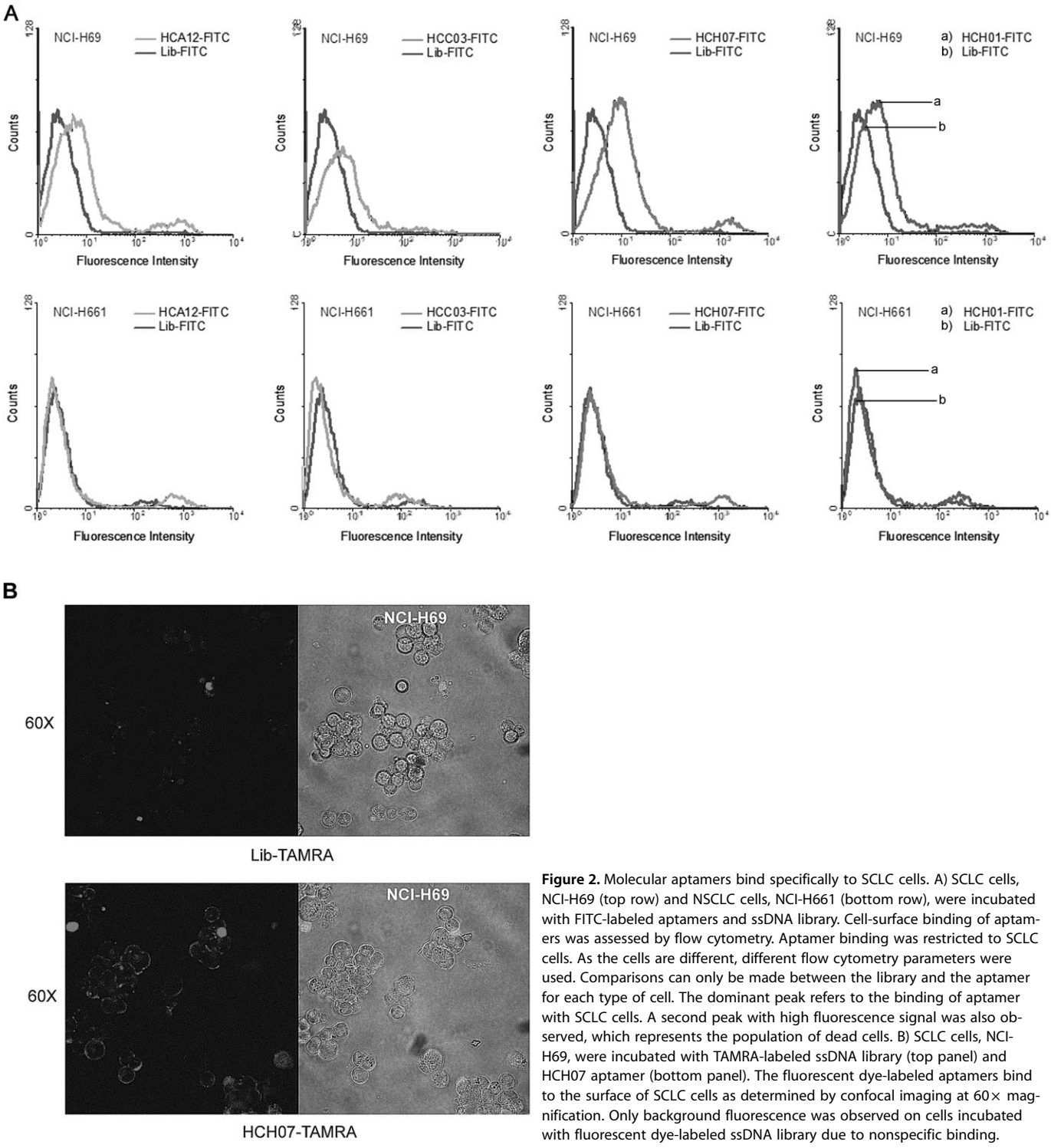


**Figure 2.** Molecular aptamers bind specifically to SCLC cells. A) SCLC cells, NCI-H69 (top row) and NSCLC cells, NCI-H661 (bottom row), were incubated with FITC-labeled aptamers and ssDNA library. Cell-surface binding of aptamers was assessed by flow cytometry. Aptamer binding was restricted to SCLC cells. As the cells are different, different flow cytometry parameters were used. Comparisons can only be made between the library and the aptamer for each type of cell. The dominant peak refers to the binding of aptamer with SCLC cells. A second peak with high fluorescence signal was also observed, which represents the population of dead cells. B) SCLC cells, NCI-H69, were incubated with TAMRA-labeled ssDNA library (top panel) and HCH07 aptamer (bottom panel). The fluorescent dye-labeled aptamers bind to the surface of SCLC cells as determined by confocal imaging at 60× magnification. Only background fluorescence was observed on cells incubated with fluorescent dye-labeled ssDNA library due to nonspecific binding.

chance for some of the selected aptamers to recognize them by the same target molecule as on SCLC cells. The addition of further negative selection criteria is expected to minimize this type of cross-reactivity.

Along with the tests with live cancer cells, it is also interesting to determine whether the aptamers developed from live cells can recognize fixed cells, which constitutes the main assay format for the retrospective analysis of preserved specimens such as sputum and biopsy in early detection studies. It will also be useful for histological examination in the clinical diagnosis of lung cancer. We processed formalin-fixed, paraffin-embedded cell line tissue arrays from SCLC and NSCLC samples. After incubation with fluorescent dye-labeled aptamers, washing, and dehydration, stained array slides were mounted for array scanning and confocal imaging. Binding of aptamer probes was found to be specific for SCLC, as only background level binding was observed for NSCLC (Figure 3 A). Notably, most aptamers bound to the periphery of target cells as

  

**Table 2.** Tests of developed aptamers with cultured cancer cell lines.

| Cultured cancer cell line | Receptors[a] | Aptamer HCA12 | HCC03 | HCH07 | HCH01 |
|---|---|---|---|---|---|
| NCI-H69 (small-cell carcinoma) | IGF II | + | + | + | + |
| NCI-H146 (small-cell carcinoma, bone marrow) | IGF II | − | + | + | + |
| NCI-H128 (small-cell carcinoma, pleural effusion) | NA | + | + | + | + |
| NCI-H661 (large-cell carcinoma, lymph node) | NA | − | − | − | − |
| NCI-H23 (adenocarcinoma) | PDGF, TGF, EGF | − | − | + | − |
| NCI-H1385 (squamous-cell carcinoma, lymph node) | NA | − | − | − | − |
| CCRF-CEM (T-cell acute lymphoblastic leukemia) | NA | − | − | − | − |
| Ramos (B-cell human Burkitt's lymphoma) | NA | − | − | − | − |
| IMEA (liver cancer) | NA | − | − | + | − |
| BNL (liver cancer) | NA | − | − | + | − |

[a] NA = information not available.

shown in magnified confocal microscopy images (Figure 3B and Figure S4). This binding pattern is similar to that observed in tests of live cells, and further confirmed that aptamers indeed bind to their target molecules on fixed cells. These data indicate that specific recognition of cell line tissue array by aptamers is dependent on the presence of cell-surface markers, which are still biochemically active after fixing cells.

### Clinical sample tests and detection of SCLC cells in whole blood samples

Next, we assessed the sensitivity and specificity of the aptamers for their ability to detect cancer cells in a clinical sample from a patient of SCLC. A substantial change in fluorescence intensity was noted in the SCLC patient's sample after incubation with dye-labeled aptamers, indicating that aptamers developed for cultured cells are also able to recognize SCLC cells from clinical samples (Figure 4A). This result clearly demonstrates the applicability of these aptamers to clinical samples, an important prerequisite for the successful detection of SCLC cells in complex biological matrixes.

We also sought to determine whether aptamers retain the ability to specifically recognize SCLC cells in the environment of human blood, another criterion for the application of aptamers in clinical tests. The binding of fluorescently labeled aptamers to SCLC cells mixed with human whole blood was assessed by flow cytometry. As controls, we also tested aptamers with human whole blood and SCLC cells in buffer. Aptamer specificity in human whole blood is consistent with results obtained in buffer experiments. SCLC cells were recognized by aptamers specifically, and no interference from various blood cells was observed (Figure 4B). Regarding their stability in human blood, it was found that aptamer modification with nonnatural nucleic acids can significantly improve half-life without compromising binding ability.[27] These results, together with the patient sample results, indicate the potential for developed aptamer probes to be used in clinical tests.

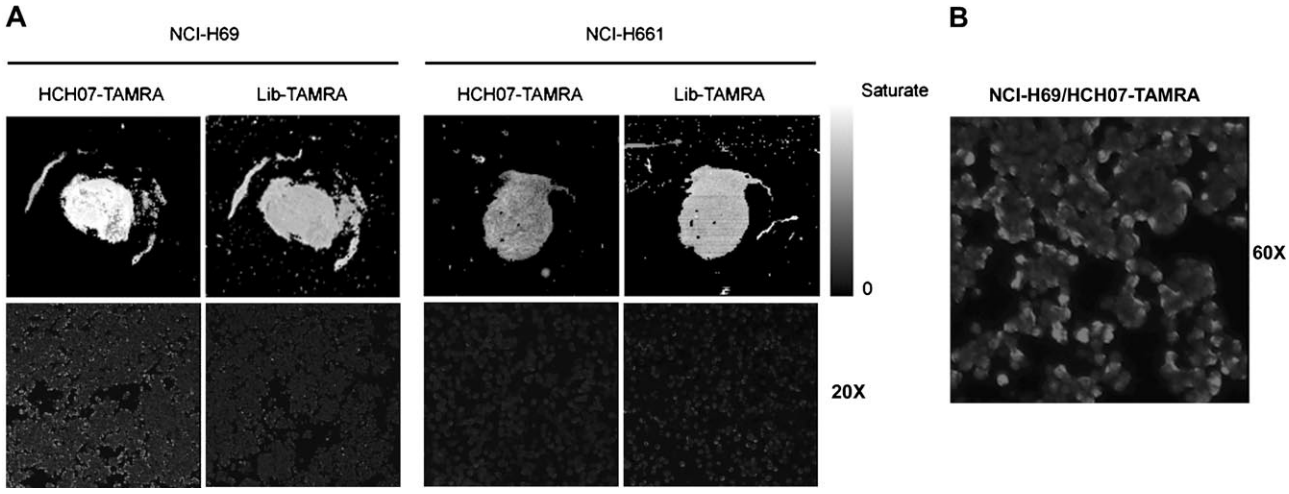


**Figure 3.** Specific binding of molecular aptamers to formalin-fixed, paraffin-embedded SCLC cell line tissue arrays. A) Recognition of SCLC and NSCLC cell line tissue arrays by fluorescent dye-labeled aptamer. Sections of formalin-fixed SCLC and NSCLC cells embedded in paraffin were stained with TAMRA-labeled aptamer and analyzed by array scanning (top row) and confocal imaging at 20× magnification (bottom row). TAMRA-labeled ssDNA library was used as a control. Binding of aptamer is specific to the SCLC cell line tissue array. B) Fluorescent aptamer-stained SCLC cell line tissue array shows similar binding pattern to live cells by magnified confocal imaging (60× magnification). Note the binding of fluorescent dye-labeled aptamers to the periphery of fixed SCLC cells. Confocal image of fluorescent ssDNA library as control is shown in Figure S4 of the Supporting Information.

  

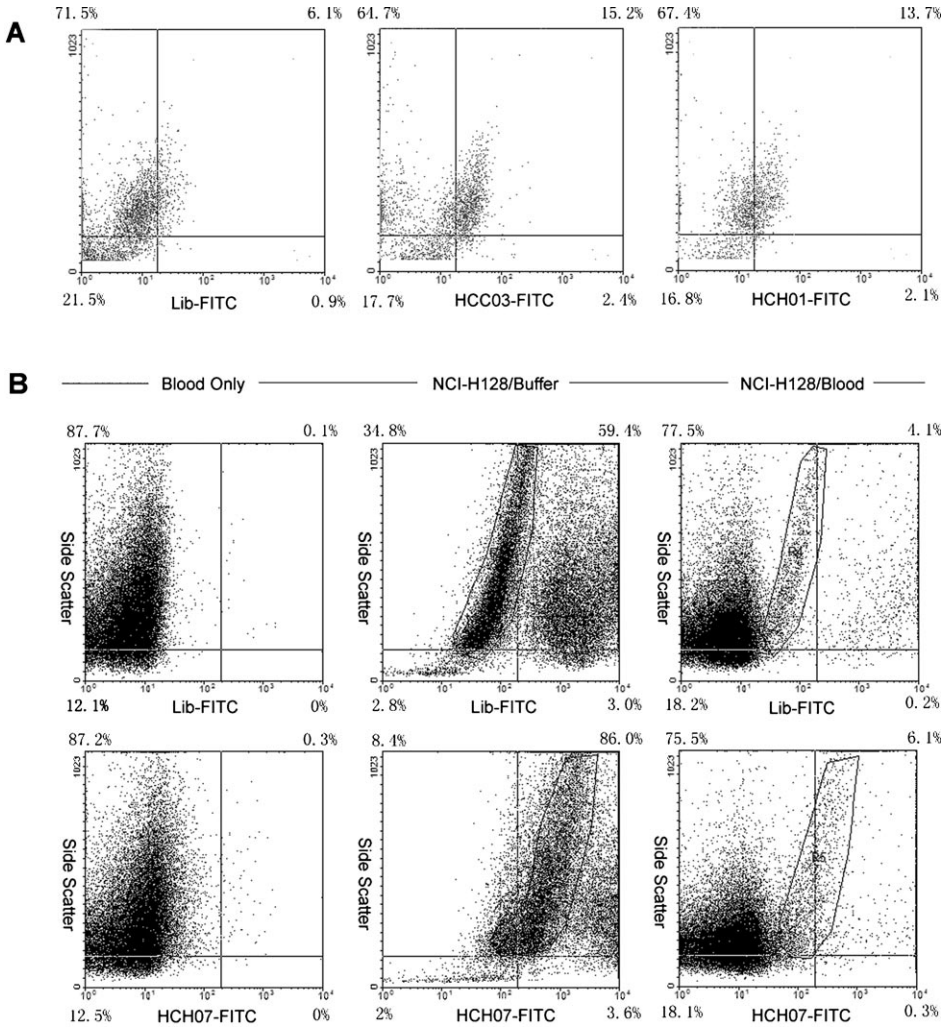


**Figure 4.** Recognition of SCLC cells in a patient sample and human whole blood by molecular aptamers. A) Clinical samples from a patient of SCLC were incubated with either FITC-labeled ssDNA library or FITC-labeled aptamers (HCC03 and HCH01). Samples were then analyzed by flow cytometry. SCLC cells were clearly identified in the patient sample by aptamer probes. B) Detection of SCLC cells in whole blood with aptamers. In human whole blood, blood cells were incubated with FITC-labeled aptamer and ssDNA library as a control for specificity (left panels). SCLC cells in buffer were incubated with FITC-labeled aptamer and ssDNA library to compare with SCLC cells in whole blood as a positive control (middle panels). Circled regions are live SCLC cells. The binding of aptamer to SCLC cells in human whole blood was also assessed (right panels). Circled regions are live SCLC cells. The aptamers retain specificity to SCLC cells in human whole blood as determined by flow cytometry. No interference from blood cells was observed.

### Extraction and detection of SCLC cells with aptamer-conjugated nanoparticles

During the early stages of lung cancer, malignant lesions begin to shed circulating cells. Encoding valuable information for prognosis, these exfoliated cells should be of great value for early lung cancer detection. Previously, the enrichment and detection of rare exfoliated cells were mainly performed by flow sorting and immunomagnetic cell sorting, techniques that suffer from poor sensitivity and specificity.[28,29] To evaluate the potential of the selected aptamers for early lung cancer detection, we prepared aptamer-conjugated magnetic nanoparticles and aptamer-conjugated fluorescent nanoparticles to isolate, enrich, and detect rare SCLC cells with a method previously established in our research group.[30] The spiked tumor cells were first incubated with aptamer-conjugated magnetic and fluorescent nanoparticles. Magnetic nanoparticle-bound cells were then isolated by magnetic separation. After recovery, we measured the fluorescence of the dye-doped nanoparticles, which also bound to the isolated cells through aptamers. Whereas two different SCLC cell types were effectively isolated and detected, the extraction of NSCLC cells was very inefficient (Figure 5 A). Additionally, very low background fluorescence signal was observed in the control experiment with DNA library conjugated nanoparticles, suggesting that nonspecific extraction of tumor cells is rare with this method. Effective enrichment and detection of SCLC cells were verified by confocal imaging results, which showed that the extracted tumor cells indeed bound to aptamer-conjugated nanoparticles (Figure 5 B). Moreover, the dye-doped nanoparticles confer great sensitivity to the detection of extracted rare tumor cells. Therefore, this aptamer-conjugated nanoparticle approach has the capacity to enrich and detect rare lung cancer cells, which is critical for early diagnosis of lung cancer.

## Conclusions

We have developed a panel of aptamer probes with specificity for SCLC cells by using cell-SELEX. SCLC was chosen as the model system for its worst prognosis among all lung cancer subtypes. The molecular differences among various cell types resulted in the evolution of specific aptamer probes. Aptamers developed in this way specifically recognize SCLC cells, whereas NSCLC cells are not recognized. The target molecules of these aptamers are therefore reasoned to be preferentially expressed in SCLC. The interaction between aptamers and their target molecules were further characterized and confirmed. We found that aptamers indeed bind to their target molecules on the cell surface, and this interaction can be affected by treating cells with protease. Moreover, it was shown that the cell-SELEX strategy can be used as an effective tool to select molecular probes for specific SCLC recognition. The method can be used

  

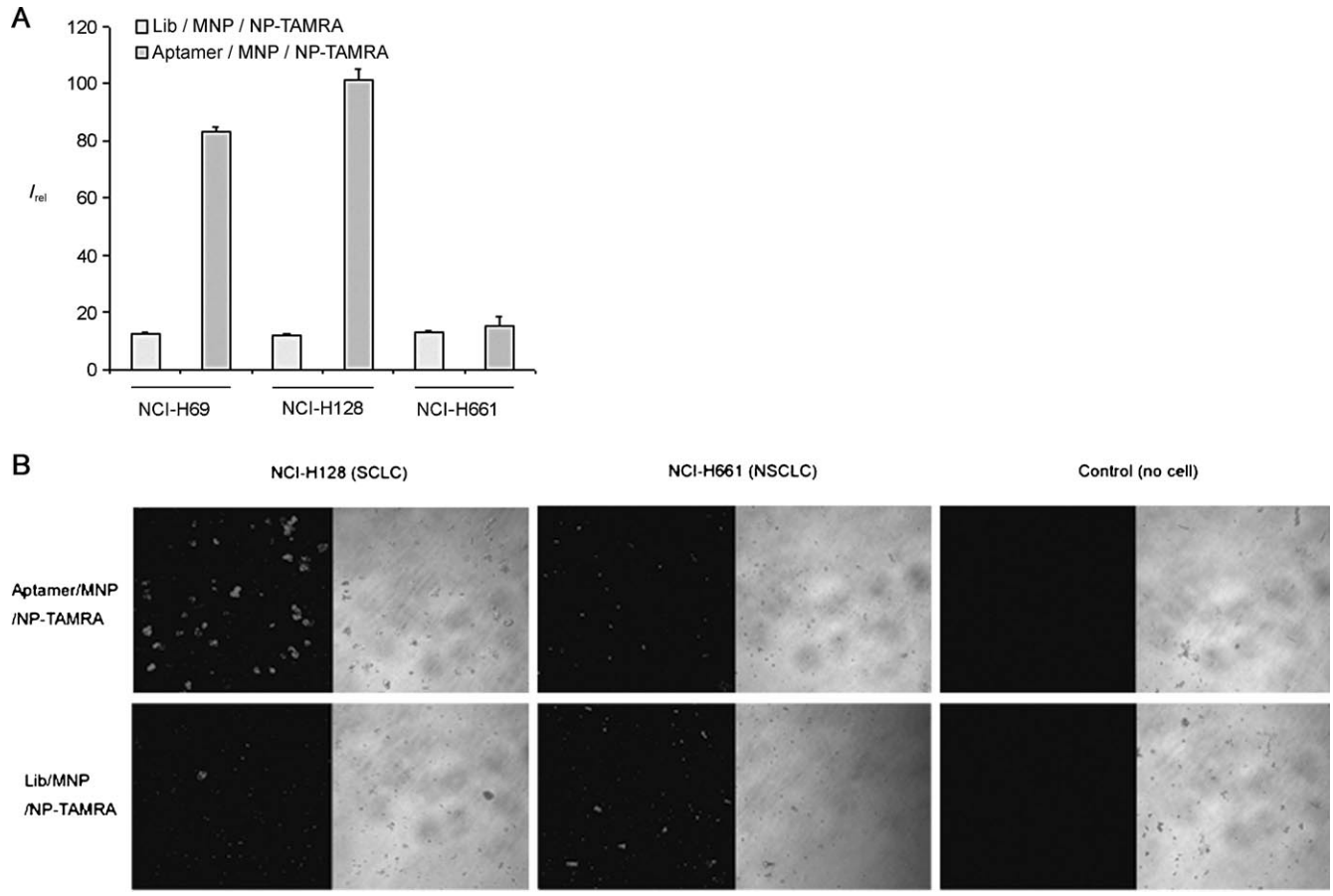


**Figure 5.** Extraction and detection of SCLC cells for enrichment and early diagnosis with aptamer-conjugated magnetic/fluorescent nanoparticles. A) Equivalent amounts of the spiked SCLC (NCI-H69 and NCI-H128) and NSCLC (NCI-H661) cells were incubated with aptamer-conjugated magnetic and fluorescent nanoparticles. Aptamer-bound cells were subsequently isolated by magnetic separation. Total extracted cell quantity after magnetic separation was determined by measuring the fluorescence signal of dye-doped nanoparticles that also bind to cells ($I_{rel}$ = relative fluorescence intensity of cells extracted by aptamer-linked nanoparticles). Aptamer-conjugated nanoparticles effectively extracted SCLC cells but not NSCLC cells (darker gray bars); ssDNA library-conjugated nanoparticles were used in control experiments and showed only limited nonspecific extraction of tumor cells (light gray bars). B) Confocal imaging of cells extracted by aptamer-conjugated nanoparticles (top row) and ssDNA library-conjugated nanoparticles (bottom row). Extraction and detection of SCLC cells with this aptamer-based approach were verified.

for both cell suspensions as previously demonstrated and cell aggregates, the typical morphology of SCLC.

Our expanded screening results show that the aptamers generated for certain SCLC cell lines are also able to recognize other SCLC cell lines of the same type, but seldom bind to other lung cancer subtypes or other types of cancer (such as leukemia or liver cancer). This suggests that the developed aptamer probes have the potential for practical use with clinical samples. Moreover, the aptamers developed from live cells can also recognize fixed cells, the main assay format for retrospective analysis of preserved specimens for early detection, as well as histological examination in clinical diagnosis of lung cancer. Notably, these aptamers exhibited the same specificity for cancer cells from SCLC patients as they did with cultured cells. In complex biological environments such as human whole blood, the specific binding capacity of the aptamers was not compromised. These results indicate that developed aptamer probes could be appropriate for use in clinical tests.

In this study we also tested aptamers for possible application in the early detection of lung cancer, particularly enrichment and detection of exfoliated tumor cells, by using aptamer-conjugated magnetic nanoparticles and fluorescent nanoparticles. The high affinity and great specificity of aptamers resulted in effective extraction of SCLC cells by magnetic separation, and the dye-doped nanoparticles allowed sensitive detection after cell extraction. Thus, the aptamer-conjugated nanoparticle strategy may substantially improve the efficiency of detecting circulating tumor cells, thereby potentially facilitating the early detection of lung cancer.

Early detection and local therapy are widely believed to decrease the overall mortality related with lung cancer, especially SCLC, by halting or reversing the progression of pre-malignant lesions at an early stage. Whereas various imaging-based methods have been described for early detection, most of these methods had only limited effects on cancer mortality due to their relatively low sensitivity. To improve this situation, molecular approaches have been explored as a means for detection through specific molecular markers. However, these molecular-marker-based techniques also showed unsatisfactory results. For example, among more than 100 monoclonal anti-

  

bodies for SCLC and NSCLC,[31] none of their antigens is exclusively expressed in SCLC samples. Therefore, the antibodies used for the early detection of lung cancer do not have sufficient specificity and may cross-react with normal, mildly atypical, moderately atypical exfoliated epithelial cells, and even normal bronchial epithelium. Furthermore, the availability of antibodies is limited to those characterized in previous studies.

In this study, developed aptamers showed great specificity for SCLC but not NSCLC. This is because these aptamers were generated based on the molecular differences between the two lung cancer subtypes by cell-SELEX. Whether the specificity of these aptamers would eventually prevent cross-reactivity and generate fewer false positives in actual early detection tests needs to be studied in greater detail. We have shown that aptamers developed in this work are suitable for multiple types of early detection studies. First, retrospective analysis of preserved specimens could be done with these aptamers by using assay formats such as flow cytometry and confocal imaging as demonstrated herein. Second, aptamer-conjugated nanoparticles are able to isolate, enrich, and detect exfoliated tumor cells in peripheral blood. These aptamers could also be very useful for lung cancer subtyping during screening[32] and planning appropriate treatment, for example, in avoiding excessive therapy for cases of resectable NSCLC.

With cell-SELEX, molecular aptamers can be readily developed for any cancer cells of interest without prior knowledge of cell-surface marker proteins, and are thus more flexible and practical to use than other molecular-marker-based methods. It is noteworthy that a panel of aptamer probes for multiple cell-surface marker differentiation can be developed by our strategy. The combination of multiple markers will ultimately be more accurate and predictive than the single-marker strategy mainly used in previous studies. An further advantage of this aptamer-based approach is that molecular markers are recognized in their native state on living cell surfaces. The molecular aptamers also may have important advantages over other methods for early lung cancer detection in terms of sensitivity, reproducibility, simplicity, robustness, production, and flexibility regarding modification. When coupled with appropriate assay formats, aptamers show great potential for use in clinical tests.

The benefits of this aptamer approach for the early detection of lung cancer remains to be determined in a prospective trial. It may also be able to detect pre-invasive lesions even before the malignant cells have exfoliated, when local therapy has limited effect. In addition, it could indicate a possible relapse in the early stage[33] for proper adjustment in therapy if specific cell-surface markers can be identified. This approach would also provide valuable information for the understanding of progressive neoplastic differentiation of lung cancer during the early stage. However, the use of aptamers for lung cancer screening and whether they can eventually decrease overall mortality must first be determined in a randomized clinical trial.

The evaluation of additional aptamers selected by cell-SELEX with high affinity and specificity will also be continued. Meanwhile, the identification of biologically relevant cell-surface markers that can be recognized by aptamers is under investigation.[34,35] In conjunction with further selection of aptamers, biomarker identification, and assay development, the development of new drugs and novel targeted drug-delivery methods may be accelerated in the future. The proteomics presented by the aptamer approach together with genomics, molecular imaging, and clinical factors, will in principle achieve the molecular profiling of lung cancer and provide tailored treatments, thereby realizing personalized medicine.[36]

## Experimental Section

**Materials:** Unless otherwise noted, all chemicals were purchased from Sigma–Aldrich and Fisher Scientific.

**Buffers:** Washing buffer was prepared by dissolving glucose (4.5 $g\,L^{-1}$), $MgCl_2$ (5 mM), and bovine serum albumin (1 $mg\,mL^{-1}$) in Dulbecco's PBS (pH 7.3). Yeast tRNA (0.1 $mg\,mL^{-1}$) was added in washing buffer to prepare binding buffer with minimal nonspecific binding.

**Cell culture:** NCI-H69 (small-cell carcinoma), NCI-H661 (large-cell carcinoma), NCI-H146 (small-cell carcinoma), NCI-H128 (small-cell carcinoma), NCI-H23 (adenocarcinoma), NCI-H1385 (squamous-cell carcinoma), CCRF-CEM (T-cell acute lymphoblastic leukemia), and Ramos (B-cell human Burkitt's lymphoma) cells were purchased from American Type Culture Collection (ATCC), and maintained at 37 °C and 5% $CO_2$ in RPMI 1640 medium (ATCC) supplemented with 10% heat-inactivated FBS (GIBCO) and penicillin–streptomycin (100 $U\,mL^{-1}$, Cellgro). IMEA (liver cancer) and BNL (liver cancer) cells were obtained from the Department of Pathology at the University of Florida.

**DNA synthesis and purification:** An ABI 3400 DNA synthesizer (Applied Biosystems) was used for the synthesis of the single-stranded (ss) DNA library (71-mers containing a randomized 35-nucleotide region and two primer binding sites: 5′-TAC CAG TGC GAT GCT CAG $(N)_{35}$ CTG ACG CAT TCG GTT GAC-3′), PCR primers, and selected aptamers. The products were further purified by HPLC (ProStar, Varian) using a $C_{18}$ column (Econosil, 5 U, 250×4.6 mm, Alltech Associates) and a linear elution gradient. The HPLC-purified product was then dried, trityl group deprotected, and resuspended in buffer for use. UV/Vis measurements were performed with a Cary Bio-300 UV spectrometer (Varian) for DNA quantitation.

**Cell-SELEX:** Target cells (NCI-H69) and control cells (NCI-H661) were counted and tested for viability before experiments. The ssDNA library (10 nmol in 1 mL binding buffer) was first denatured at 95 °C for 5 min and kept on ice for 10 min. Target cells ($2\times10^6$) were washed, dissociated (0.53 mM EDTA/PBS), and then incubated with the ssDNA library at 4 °C for 30 min. After washing, the cell-bound DNAs were eluted to 300 μL binding buffer by heating at 95 °C for 5 min. The eluted DNAs were further incubated with excess control cells at 4 °C for 30 min for counter-selection (eliminated in the first round of selection). After counter-selection, the DNAs that did not bind to control cells were collected, desalted, and PCR-amplified with FITC- and biotin-labeled primers. The PCR product of the first round of selection was then processed to generate ssDNAs for the next round of selection. For the second round of selection, all product of the first round was dissolved in 200 μL binding buffer as the starting ssDNA pool. To increase the stringency of selection, the washing strength was enhanced by gradually increasing washing time (from 1 to 10 min), washing volume (from 1 to 3 mL), and washing cycles (from 3 to 5 times). The SELEX progress was monitored by flow cytometry.

  

**Real-time PCR:** At the end of every round of selection, target-cell-specific DNA molecules were PCR amplified to form the starting pool for the next round of selection. Real-time PCR was first performed to determine the amount of DNA molecules to be amplified, using iTaq DNA polymerase (Bio-Rad) and a MyiQ real-time PCR system (Bio-Rad). SYBR green (Molecular Probes) was used for the detection of PCR products. PCR cycles were then optimized according to the template amount. The bulk of target-cell-specific DNA molecules was finally PCR amplified with the optimized PCR conditions. Primers for PCR amplification are:

forward primer: 5′-TACCAGTGCGATGCTCAG-3′

reverse primer: 5′-GTCAACCGAATGCGTCAG-3′

Unlabeled forward and reverse primers were used for real-time PCR detection with SYBR green. FITC-labeled forward primer and triple-biotinylated (trB) reverse primer were used to generate PCR product for flow cytometry assays. TAMRA-labeled forward primer and triple-biotinylated (trB) reverse primer were used to generate PCR product for confocal imaging. PCR parameters consisted of Taq activation (3 min at 95 °C), and 15 cycles of PCR at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 15 s, followed by 5 min of extension at 72 °C. Standard curves were generated for real-time PCR. Specificity of PCR amplification was verified by melt curve analysis. Amplification products were also resolved by agarose gel electrophoresis and visualized by ethidium bromide staining.

**Single-stranded DNA generation:** To generate ssDNA from PCR product for subsequent selection rounds, the sense strand was separated from the biotinylated antisense strand by streptavidin-coated Sepharose beads (Amersham Pharmacia Biosciences). After elution with alkaline solution (0.2 M NaOH), the sense strand was desalted with a Sephadex G-25 column (NAP-5, Amersham Pharmacia Biosciences), quantified by UV/Vis, and dried by rotary evaporation (SpeedVac). The product was then resuspended in buffer to be used for the next round of selection.

**Molecular cloning:** To isolate individual aptamers from the selected pool, cloning was performed after 25 rounds of selection. The most selected ssDNA pool was PCR amplified with unlabeled primers and inserted into the pCR 2.1-TOPO TA cloning vector (Invitrogen). The vector was then transformed into *E. coli*. Individual cultured colonies were picked to extract the plasmids for sequencing.

**Sequencing:** Cloned sequences were determined with 454 Life Sciences DNA sequencing unit, GS20, at the Interdisciplinary Center for Biotechnology Research (ICBR) of the University of Florida.

**Multiple sequence alignment analysis:** The sequencing results were subjected to multiple sequence alignment analysis with the MEME/MAST System, version 3.5.3 (developed by Timothy Bailey, Charles Elkan, and Bill Noble at the UCSD Computer Science and Engineering department with input from Michael Gribskov at Purdue University, http://meme.nbcr.net) to discover highly conserved motifs in groups of selected DNA sequences. The discovered consensus sequences with high repeats among selected pools were then synthesized and tested for specificity and affinity.

**Flow cytometry:** To monitor the enrichment of aptamers along with the progress of SELEX, FITC-labeled ssDNA pools were incubated with NCI-H69 or NCI-H661 cells ($1\times10^6$) in 400 μL binding buffer at 4 °C for 30 min. Cells were washed twice after incubation and analyzed by flow cytometry. The binding of selected aptamers to SCLC cells, NSCLC cells, leukemia cells, and liver cancer cells were similarly analyzed. Flow cytometry was performed on a FACScan cytometer with CellQuest software (Becton Dickinson).

**Confocal imaging:** The binding of selected ssDNA pools and individual aptamers to SCLC cells was evaluated by fluorescence confocal imaging. Cells were incubated with TAMRA-labeled aptamers (250 nM) in 100 μL binding buffer at 4 °C for 30 min. After washing, cell suspension (20 μL) was dropped on a covered glass slide for examination with a confocal microscope. Fluorescence confocal imaging was performed on a Fluoview 500/IX81 inverted confocal scanning microscope system (Olympus). A 5-mW, 543-nm He–Ne laser was used as the excitation source for the TAMRA dye. The objective used for imaging was a 60× oil-immersion objective (PLAPO 60× O3PH) with a numerical aperture of 1.40 (Olympus). A 20× objective with a numerical aperture of 0.7 (Olympus) was also used for large-field imaging. The staining of cell line tissue arrays by fluorescent aptamers and extraction of SCLC cells by aptamer-conjugated nanoparticles were evaluated by confocal imaging as described above.

**Saturation analysis:** Saturation analysis was performed to measure the relative cell-surface binding affinities of developed aptamers. Cells were incubated with FITC-labeled aptamers at 4 °C for 30 min, washed three times with 400 μL washing buffer, and finally resuspended in 400 μL binding buffer containing 20% FBS. Cells were then assayed by flow cytometry. Concentrations of FITC-labeled aptamers for the relative affinity measurements varied from 0 to 1 μM. The FITC-labeled ssDNA library was used to determine nonspecific binding. The mean fluorescence intensity of aptamer-bound cells (nonspecific binding of DNA library subtracted) was used to calculate the fraction of bound aptamer at various concentrations. All affinity measurements were performed in triplicate. The results are described as mean ± SEM. The equilibrium dissociation constants ($K_d$) were obtained by fitting the cell-surface binding data of aptamers to a one-site saturation model with SigmaPlot 9.0 (Jandel Scientific).

**Enzymatic treatment:** To verify the binding of aptamers to SCLC cell-surface markers, cells were examined by enzymatic treatment. Cells ($1\times10^6$) were washed with PBS (1 mL) and treated with 200 μL of 0.05% trypsin/0.53 mM EDTA in HBSS (Fisher Biotech) or 0.1 mg mL$^{-1}$ proteinase K (Fisher Biotech) in PBS at 37 °C for 2 min. FBS was then added to quench the enzyme activity. After washing with binding buffer, the cells were analyzed for aptamer binding with flow cytometry and confocal imaging as described above.

**Cell line tissue array:** Cultured SCLC and NSCLC cell lines were processed into homogeneous tissue arrays to evaluate the binding of aptamers to fixed cells. All cell line tissue arrays were prepared at the University of Florida Diagnostic Reference Laboratories. Cells ($10\times10^6$) grown in culture were first prepared as a cell suspension in a minimal amount of medium (adherent cells were detached by trypsin/EDTA treatment). Cells were then fixed with 4% formaldehyde and mixed with 1% agarose in iso-osmotic PBS. The solidified cell blocks were cut into serial sections and processed on paraffin-embedded slides. Prepared cell line tissue arrays were stained with hematoxylin and eosin (H&E) for quality control.

**Cell line tissue array staining:** Cell line tissue arrays were first treated with xylene and ethanol (100%, 95%, and 70%) to remove the paraffin. For antigen retrieval, the dried tissue arrays were rinsed with PBS and kept in 1 mM EDTA/Tris buffer (pH 8.0) at 95 °C for 15 min. Tissue arrays were then incubated with TAMRA-labeled aptamers (200 μL, 0.25 μM) in binding buffer at 4 °C for 30 min. After washing and dehydration, the stained array slides were mounted for evaluation. Aptamer staining of cell line tissue arrays

  

were analyzed by array scanning and confocal imaging. For array scanning, the stained array slides were scanned into a computer with a microarray scanner (2100 BioAnalyzer, Agilent) at a scan resolution of 10 μm and analyzed with Agilent G2567AA Feature Extraction software (v.9.1). To confirm the array scanning results and to show the binding details, the same stained array slides were imaged with an FV500-IX81 confocal microscope (Olympus) with excitation at $\lambda = 543$ nm. Images were collected with both 60× and 20× objectives as described above.

**Clinical sample test:** SCLC patient samples were obtained from the Department of Pathology at the University of Florida. Cells were washed and counted for incubation with aptamers. Cell-surface binding of FITC-labeled aptamers was analyzed by flow cytometry as detailed above.

**Binding assay in human whole blood:** To evaluate the binding capacity of aptamers in a complex biological environment, SCLC cells ($2\times10^6$) were prepared as described above and mixed with 3.5 μL human whole blood (IPLA-WB1, Innovative Research, Inc.) in 300 μL buffer. Human whole blood was prepared by mixing with sodium heparin as anticoagulant. FITC-labeled aptamers (100 μL, 1 μM) were added to SCLC cells previously spiked in human whole blood. After incubation at 4 °C and thorough washing, the binding of aptamers to SCLC cells in blood was assessed with flow cytometry. For controls, human whole blood and cells in buffer were incubated with aptamers and analyzed by flow cytometry. Background binding of aptamers to blood cells was negligible.

**Aptamer-conjugated magnetic and fluorescent nanoparticles:** For the synthesis of aptamer-conjugated magnetic nanoparticles, iron oxide-doped magnetic nanoparticles (65 nm) were first prepared by precipitating iron oxide as previously described.[30] The magnetite core particles were then coated with silica by the hydrolysis of TEOS, and treated with TEOS. After washing, avidin coating was performed by incubating a solution of silica-coated magnetic nanoparticles (0.1 mg mL$^{-1}$) with avidin (5 mg mL$^{-1}$) at 4 °C for 12 h. The avidin-coated magnetic nanoparticles were then washed with PBS and stabilized by cross-linking with glutaraldehyde (1%) at 25 °C for 1 hour. After washing with Tris-HCl buffer, the avidin-coated magnetic nanoparticles (0.2 mg mL$^{-1}$) were incubated with excess biotinylated DNA aptamers and ssDNA library at 4 °C for 12 h. The prepared aptamer-conjugated magnetic nanoparticles were washed and stored at a final concentration of 0.2 mg mL$^{-1}$ at 4 °C for use. For the synthesis of aptamer-conjugated fluorescent nanoparticles, TAMRA dye-doped nanoparticles were first prepared by the reverse micro-emulsion method as previously described.[30] After silica polymerization and stabilization treatment with TEOS, the dye-doped nanoparticles were coated with avidin as described above. Avidin-coated dye-doped nanoparticles were further conjugated with excess biotinylated DNA aptamers and ssDNA library. The prepared aptamer-conjugated fluorescent nanoparticles were washed and stored at a final concentration of 10 mg mL$^{-1}$ at room temperature for use.

**Extraction and detection of SCLC cells:** For each experiment, cells ($1.0\times10^5$) were prepared as described above and dispersed in 200 μL cell media buffer. The specified amount of aptamer-conjugated magnetic and fluorescent nanoparticles was then simultaneously added to the cell suspension. After incubation and washing for 30 min, cells were isolated from cell media buffer by magnetic extraction and recovered in buffer (20 μL) for confocal imaging and fluorescence measurement. A 2-μL aliquot of the extracted sample was assessed by confocal imaging as described above. The remainders of the samples were then added to a 96-well plate, and the fluorescence of dye-doped nanoparticles bound to extracted cells was measured by a plate reader (Packard). The ssDNA library-conjugated magnetic and fluorescent nanoparticles were used for control experiments.

**Glossary**

| | |
|---|---|
| EDTA | ethylenediaminetetraacetic acid |
| FBS | fetal bovine serum |
| FITC | fluorescein isothiocyanate |
| NSCLC | non-small-cell lung cancer |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| SCLC | small-cell lung cancer |
| SELEX | systematic evolution of ligands by exponential enrichment |
| TAMRA | etramethyl-6-carboxyrhodamine |
| TEOS | tetraethoxyorthosilicate |

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[1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Q. Xu, M. J. Thun, *CA-Cancer J. Clin.* **2007**, *57*, 43–66.
[2] P. C. Hoffman, A. M. Mauer, E. E. Vokes, *Lancet* **2000**, *355*, 479–485.
[3] J. L. Mulshine, D. C. Sullivan, *N. Engl. J. Med.* **2005**, *352*, 2714–2720.
[4] D. M. Jackman, B. E. Johnson, *Lancet* **2005**, *366*, 1385–1396.
[5] W. N. Rom, J. G. Hay, T. C. Lee, Y. X. Jiang, K. M. Tchou-Wong, *Am. J. Respir. Crit. Care Med.* **2000**, *161*, 1355–1367.
[6] F. R. Hirsch, W. A. Franklin, A. F. Gazdar, P. A. Bunn, *Clin. Cancer Res.* **2001**, *7*, 5–22.
[7] A. McWilliams, C. MacAulay, A. F. Gazdar, S. Lam, *Oncogene* **2002**, *21*, 6949–6959.
[8] J. L. Mulshine, *Nat. Rev. Cancer* **2003**, *3*, 65–73.
[9] E. F. Patz, P. C. Goodman, G. Bepler, *N. Engl. J. Med.* **2000**, *343*, 1627–1633.
[10] S. D. Bernal, A. D. Elias, *J. Clin. Oncol.* **1988**, *6*, 1676–1678.
[11] I. I. Wistuba, J. Berry, C. Behrens, A. Maitra, N. Shivapurkar, S. Milchgrub, B. Mackay, J. D. Minna, A. F. Gazdar, *Clin. Cancer Res.* **2000**, *6*, 2604–2610.
[12] L. Mao, *Oncogene* **2002**, *21*, 6960–6969.
[13] L. Mao, R. H. Hruban, J. O. Boyle, M. Tockman, D. Sidransky, *Cancer Res.* **1994**, *54*, 1634–1637.
[14] N. A. Levin, P. M. Brzoska, M. L. Warnock, J. W. Gray, M. F. Christman, *Genes Chromosomes Cancer* **1995**, *13*, 175–185.
[15] M. S. Tockman, P. K. Gupta, J. D. Myers, J. K. Frost, S. B. Baylin, E. B. Gold, A. M. Chase, P. H. Wilkinson, J. L. Mulshine, *J. Clin. Oncol.* **1988**, *6*, 1685–1693.
[16] K. Yanagisawa, Y. Shyr, B. G. J. Xu, P. P. Massion, P. H. Larsen, B. C. White, J. R. Roberts, M. Edgerton, A. Gonzalez, S. Nadaf, J. H. Moore, R. M. Caprioli, D. P. Carbone, *Lancet* **2003**, *362*, 433–439.
[17] T. D. Chanin, D. T. Merrick, W. A. Franklin, F. R. Hirsch, *Curr. Opin. Pulm. Med.* **2004**, *10*, 242–247.
[18] M. Blank, T. Weinschenk, M. Priemer, H. Schluesener, *J. Biol. Chem.* **2001**, *276*, 16464–16468.
[19] D. A. Daniels, H. Chen, B. J. Hicke, K. M. Swiderek, L. Gold, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15416–15421.

  

[20] D. Shangguan, Y. Li, Z. W. Tang, Z. H. C. Cao, H. W. Chen, P. Mallikaratchy, K. Sefah, C. Y. J. Yang, W. H. Tan, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11838–11843.
[21] D. N. Carney, *Lancet* **1992**, *339*, 843–846.
[22] D. S. Ettinger, J. Aisner, *J. Clin. Oncol.* **2006**, *24*, 4526–4527.
[23] S. E. Osborne, A. D. Ellington, *Chem. Rev.* **1997**, *97*, 349–370.
[24] D. S. Wilson, J. W. Szostak, *Annu. Rev. Biochem.* **1999**, *68*, 611–647.
[25] M. Famulok, G. Mayer, M. Blind, *Acc. Chem. Res.* **2000**, *33*, 591–599.
[26] Z. W. Tang, D. H. Shangguan, K. M. Wang, H. Shi, K. Sefah, P. Mallikaratchy, H. W. Chen, Y. Li, W. H. Tan, *Anal. Chem.* **2007**, *79*, 4900–4907.
[27] D. Shangguan, Z. W. Tang, P. Mallikaratchy, Z. Y. Xiao, W. H. Tan, *ChemBioChem* **2007**, *8*, 603–606.
[28] P. S. Kraemer, C. A. Sanchez, G. E. Goodman, J. Jett, P. S. Rabinovitch, B. J. Reid, *Cytometry Part A* **2004**, *60*, 1–7.
[29] H. Iinuma, K. Okinaga, M. Adachi, K. Suda, T. Sekine, K. Sakagawa, Y. Baba, J. Tamura, H. Kumagai, A. Ida, *Int. J. Cancer* **2000**, *89*, 337–344.
[30] J. K. Herr, J. E. Smith, C. D. Medley, D. H. Shangguan, W. H. Tan, *Anal. Chem.* **2006**, *78*, 2918–2924.
[31] R. L. Souhami, P. C. L. Beverley, L. G. Bobrow, *Lancet* **1987**, *330*, 325–326.
[32] L. A. Doyle, D. Giangiulo, A. Hussain, H. J. Park, R. W. C. Yen, M. Borges, *Cancer Res.* **1989**, *49*, 6745–6751.
[33] H. Y. Chen, S. L. Yu, C. H. Chen, G. C. Chang, C. Y. Chen, A. Yuan, C. L. Cheng, C. H. Wang, H. J. Terng, S. F. Kao, W. K. Chan, H. N. Li, C. C. Liu, S. Singh, W. J. Chen, J. J. W. Chen, P. C. Yang, *N. Engl. J. Med.* **2007**, *356*, 11–20.
[34] D. H. Shangguan, Z. H. Cao, L. Meng, P. Mallikaratchy, K. Sefah, H. Wang, Y. Li, W. H. Tan, *J. Proteome Res.* **2008**, in press.
[35] P. Mallikaratchy, Z. W. Tang, K. Sefah, L. Meng, D. H. Shangguan, W. H. Tan, *Mol. Cell. Proteomics* **2007**, *6*, 2230–2238.
[36] R. S. Herbst, S. M. Lippman, *N. Engl. J. Med.* **2007**, *356*, 76–78.

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