

Aptamer-Based Capture of CXCR4-Positive Circulating Tumor Cells for Postoperative Recurrence and Metastasis Risk Assessment in Non-Small Cell Lung Cancer

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Abstract

Background: Postoperative recurrence and metastasis remain major challenges in non-small cell lung cancer (NSCLC). CXCR4-positive circulating tumor cells (CTCs) are thought to promote metastatic seeding and may serve as early biomarkers of relapse, yet their rarity in blood necessitates highly efficient isolation methods.

Methods: We developed a CXCR4-specific aptamer via magnetic bead-based SELEX and validated its binding affinity by flow cytometry. A spiral inertial microfluidic chip was then integrated with the aptamer to enable sequential size-based and affinity-based enrichment of CXCR4⁺ CTCs from peripheral blood of NSCLC patients after radical resection. The isolated cells were characterized by multi-omics analysis, and their correlation with clinical outcomes was prospectively evaluated.

Results: A high-affinity aptamer was successfully selected after five SELEX rounds. The microfluidic platform achieved efficient CTC capture with high viability. CXCR4⁺ CTC counts dynamically increased prior to radiologically detectable recurrence in a subset of patients, and their levels correlated with shorter progression-free survival.

Conclusions: The aptamer-based microfluidic strategy enables sensitive detection of CXCR4⁺ CTCs and holds promise for early risk stratification and surveillance of postoperative recurrence in NSCLC.

Introduction

Lung cancer remains the leading cause of cancer-related mortality worldwide, with non-small cell lung cancer (NSCLC) accounting for the majority of cases. Despite curative intent surgery, 30–55% of patients experience postoperative recurrence or metastasis, leading to treatment failure [1]. Postoperative pathological stage strongly correlates with survival: the 5-year overall survival rates for stage I, II, and III NSCLC patients are 77%, 50%, and 35%, respectively [2]. Current clinical surveillance tools—including PET-CT and serum tumor markers such as CEA and CYFRA21-1—suffer from temporal lag in detecting occult lesions [3] and limited sensitivity/specificity [4]. Therefore, identifying biomarkers that enable early, accurate prediction of postoperative recurrence is critically needed.

Circulating tumor cells (CTCs) are cells shed from primary tumors into the circulating system that retain genetic and phenotypic features of the original lesion [5]. Through epithelial-mesenchymal transition, CTCs enter circulation and colonize distant sites, initiating metastasis [6]. CTC clusters (2–50 cells) exhibit even higher metastatic seeding efficiency than single CTCs [7]. Compared to conventional monitoring methods, CTC detection offers high sensitivity and specificity, non-invasiveness, and real-time sampling [8]. However, CTCs are extremely rare in blood (0–50 cells/mL), making their efficient isolation technically challenging.

Chemokine receptor 4 (CXCR4) is a seven-transmembrane G protein-coupled receptor that binds specifically to stromal cell-derived factor 1 (SDF-1/CXCL12). This axis activates downstream signaling

pathways including PI3K/AKT, WNT, and NF- κ B, promoting tumor cell proliferation, migration, adhesion, and angiogenesis [9]. CXCR4 is overexpressed in multiple malignancies and serves as a marker for circulating tumor stem cells. Recent studies have shown that among CTC-positive pancreatic cancer patients, CXCR4 has higher expression and is closely related to tumor malignancy staging, suggesting that CXCR4-positive CTCs can serve as an independent prognostic factor for pancreatic cancer. [10]. Preclinical evidence indicates that CXCR4 antagonists enhance immunotherapy efficacy and suppress distant metastasis [11, 12].

Tumor cells that infiltrate distant tissues can persist in supportive niches, remaining quiescent while retaining stem-like properties, thereby acting as seeds for future relapse [13]. The CXCR4-CXCL12 axis is a key driver of this process: high CXCL12 expression in bone marrow, lung, and other microenvironments activates multiple pro-oncogenic pathways, promoting proliferation, inhibiting apoptosis, enhancing immune evasion, and fueling postoperative recurrence [14, 15]. Thus, detecting CXCR4 + CTCs in peripheral blood may enable risk stratification and intervention before clinical or radiological evidence of recurrence emerges.

Microfluidic chip technology has emerged as a powerful platform for rare cell isolation. Compared to conventional methods, microfluidic devices offer compact channel architecture, low sample consumption, short processing time, high separation efficiency, and portability [16]. Separation strategies can be based on physical properties (size, deformability) [17, 18], chemical recognition (surface markers via antibodies or aptamers), or their combination. Inertial spiral microfluidics, in particular, enables label-free, high-throughput CTC enrichment based on size differences, providing an ideal first-stage separation.

Aptamers are single-stranded DNA or RNA molecules selected through Systematic Evolution of Ligands by Exponential Enrichment (SELEX) that bind targets with high affinity and specificity [19]. In terms of working principle, microfluidic chips can achieve efficient separation of CTCs by utilizing physical properties such as size differences, morphology, and cell deformability [20, 21], chemical properties such as specific surface marker–antibody/aptamer binding, or a combination of both.

Aptamer-functionalized microfluidic devices have demonstrated improved CTC capture efficiency [22]. Notably, CD44—a CTC surface marker—mediates cell aggregation, and CXCR4 exposure further promotes distant dissemination [23]. Hence, combining aptamer-based CXCR4 recognition with microfluidic separation holds promise for isolating CXCR4 + CTCs and enabling early detection of lung cancer recurrence.

In this study, we aim to develop a CXCR4-specific aptamer with cholesterol modification, integrate it into a spiral inertial microfluidic chip for sequential physical and immunochemical separation of CXCR4 + CTCs from peripheral blood of NSCLC patients after radical resection, characterize the molecular features of this CTC subpopulation, and prospectively evaluate its correlation with postoperative recurrence, with the ultimate goal of establishing an early warning strategy for NSCLC recurrence and metastasis.

Method

2.1 Overview of Study Design and Analytical Workflow

To establish a comprehensive paradigm from technical development to clinical monitoring for lung cancer, we designed an integrated workflow encompassing highly efficient isolation of viable circulating tumor cells (CTCs) and their subsequent aptamer-based specific identification (Fig. 1). Initially, peripheral venous blood samples were collected, followed by controlled dilution and erythrocyte lysis. The pre-processed blood samples were subsequently processed through a custom-designed spiral microfluidic chip. This microfluidic platform leverages hydrodynamic and inertial forces to achieve label-free, high-throughput separation of viable CTCs from the complex leukocyte background. Concurrently, to engineer high-affinity molecular probes for CTC recognition, the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technique was employed. Utilizing a structurally diverse random single-stranded DNA (ssDNA) library, iterative cycles of target (CXCR4 protein) incubation, affinity screening, specific partitioning, and PCR amplification were executed. Following next-generation sequencing (NGS) and structural folding prediction, candidate aptamers capable of forming stable G-quadruplex structures were synthesized. Ultimately, these optimized aptamers were deployed as specific recognition elements for the in situ fluorescent imaging of the microfluidic-enriched CTCs. This integrated "isolation-to-detection" platform is formulated to facilitate precise molecular profiling of CTCs, thereby providing a robust technical foundation for the early diagnosis and prognostic monitoring of postoperative lung cancer recurrence.

2.2 Construction of the recombinant plasmid

The coding sequence of human CXCR4 was retrieved from the NCBI database, and the corresponding amino acid sequence was obtained from the HPA database. A histidine tag (His-tag) was added at the C-terminus. The DNA sequence was codon-optimized for mammalian expression and chemically synthesized. The optimized fragment was inserted into the pFastBac-1 plasmid, and the resulting construct was verified by sequencing. Recombinant bacmid DNA was generated and transfected into Sf9 insect cells to produce baculovirus.

2.3 Expression and purification of protein CXCR4

HEK293 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (<https://www.cellbank.org.cn/>). Cells were cultured and passaged in DMEM high-glucose medium supplemented with 10% fetal bovine serum in an incubator containing 50 mL/L CO₂ at 37°C. Cells were detached using 0.25% trypsin-EDTA and seeded (2.5×10^4 cells per well) in 48-well culture plates. At 24-h intervals, cells from three replicate wells were trypsinized and counted, and the average cell number per well was calculated. The measurement was continued for 8 consecutive days. The population doubling time was then calculated, and a growth curve was plotted. High-titer virus was then used to infect HEK293 cells for protein expression. The His-tagged CXCR4 protein was purified from the lysate using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography, eluted with imidazole buffer, and dialyzed

against phosphate-buffered saline. The purified protein was used as the target for subsequent aptamer selection.

2.4 Selection of CXCR4-Specific Aptamers (SELEX)

The identification of high-affinity aptamers against the CXCR4 protein was performed using a modified Systematic Evolution of Ligands by Exponential Enrichment (SELEX) strategy. A chemically synthesized random single-stranded DNA (ssDNA) library was employed, characterized by uniform melting temperature (T_m) across all sequences to ensure thermodynamic equilibrium during the selection process. Before each selection round, the ssDNA library was thermally denatured at 95°C for 5 min and immediately renatured at 4°C to facilitate the formation of stable secondary structures. To eliminate non-specific binders, a negative selection step was first conducted by incubating the library with counter-selection proteins immobilized on nickel-nitrilotriacetic acid (Ni-NTA) agarose beads. The pre-cleared supernatant was then transferred to carboxyl-functionalized magnetic beads (MBs) conjugated with recombinant CXCR4 protein. The mixture was incubated on a rotary shaker to reach binding equilibrium. Partitioning of target-bound sequences was achieved using a magnetic separator, followed by stringent washing to remove low-affinity species. Bound aptamers were eluted by heating the beads in ultrapure water at 100°C for 10 min. The enriched sub-library was quantified and amplified via quantitative PCR (qPCR) to generate the input for the subsequent round. This iterative process was repeated for 6–8 cycles. Enrichment progress was monitored by measuring the target-binding affinity of each round; once the binding rate reached a plateau, the cycle with the highest enrichment efficiency was selected for high-throughput sequencing. Bioinformatic analysis of the HTS data was performed using a custom pipeline. The sequences were categorized into three groups based on primer architecture: dual-tagged sequences, single-tagged sequences, and random core sequences. Following homology alignment and motif clustering, representative monoclonal candidates were selected for further affinity and specificity characterization.

Result

3.1 Plasmid Construction and Validation

The CXCR4 coding sequence was codon-optimized for expression in mammalian cells and synthesized with a C-terminal His-tag. This sequence was cloned into the pFastBac-1 plasmid (Fig. 2). The successful construction of the recombinant plasmid was confirmed by restriction enzyme digestion, which yielded fragments of the expected sizes, and by Sanger sequencing, which verified the correct sequence and orientation of the CXCR4 insert. These results ensured that no mutations were introduced during the cloning process, providing a validated template for the generation of bacmid DNA and subsequent protein expression.

3.2 Expression, Purification, and Characterization of Recombinant CXCR4

The codon-optimized CXCR4 sequence with a C-terminal His-tag was synthesized and cloned into the pFastBac-1 vector. After confirming the sequence integrity and orientation via restriction digestion and Sanger sequencing, the recombinant plasmid was used to generate bacmid DNA for expression in HEK293 cells. The His-tagged CXCR4 was purified using Ni-NTA affinity chromatography, and SDS-PAGE analysis (Fig. 3A) showed a primary band at 40–45 kDa, consistent with the glycosylated monomer. A secondary band at 80–90 kDa was also observed, suggesting the formation of stable homodimers. Both the monomeric and dimeric bands showed strong immunoreactivity in Western blot analysis using anti-His and anti-CXCR4 antibodies (Fig. 3B), confirming the molecular identity of the purified protein. The persistence of the dimeric form under denaturing conditions indicates high structural stability, providing a representative target for the subsequent selection process.

3.3 SELEX Screening and Affinity Characterization of Candidate Aptamers

A modified SELEX strategy was employed to isolate high-affinity aptamers against the purified CXCR4 protein. To ensure thermodynamic equilibrium, the initial ssDNA library was designed with a uniform melting temperature (T_m). The selection process consisted of five iterative rounds, each including thermal denaturation/renaturation, negative selection with Ni-NTA beads, and positive selection with CXCR4-conjugated magnetic beads. Bound sequences were eluted via heat treatment and amplified by qPCR for the next round. Following five rounds of enrichment, the resulting pool was subjected to high-throughput sequencing (HTS) and bioinformatic analysis, which categorized sequences into dual-tagged, single-tagged, and random groups. To evaluate the binding performance of the selected candidates, flow cytometry was performed using CXCR4 binding Ni beads. As shown in the fluorescence histograms (Fig. 4), the candidate aptamers exhibited a significant rightward shift in fluorescence intensity compared to the random library control, indicating strong binding affinity to cell-surface CXCR4. The mean fluorescence intensity (MFI) increased in a concentration-dependent manner, allowing for the calculation of equilibrium dissociation constants in the nanomolar range. These results confirm that the five-round SELEX process successfully enriched for aptamers capable of recognizing the target receptor in its native cellular conformation.

Discussion

In this study, we developed an integrated platform for the efficient isolation and molecular identification of circulating tumor cells (CTCs) by combining a spiral microfluidic chip with a customized CXCR4-targeted aptamer. We successfully expressed and purified the recombinant CXCR4 protein, which maintained its stable dimeric conformation, serving as an optimal target for in vitro selection. Through a rigorous five-round SELEX process utilizing a T_m -balanced library and dual-stage screening (Ni-NTA and magnetic beads), we identified candidate aptamers that exhibit high affinity and specificity for CXCR4-expressing cells, as validated by flow cytometry. These results demonstrate a complete technical closed-loop from target preparation to probe discovery

The methodology of SELEX has undergone significant evolution since its inception. Conventional SELEX often relies on nitrocellulose membrane filtration or simple centrifugation to partition bound and unbound sequences, which can lead to high background noise and limited enrichment efficiency [24, 25]. Our approach incorporates several strategic optimizations. By employing carboxyl-functionalized magnetic beads for target immobilization and a pre-clearing step with Ni-NTA agarose, we minimized the recovery of non-specific binders [26]. More importantly, the use of a library with a uniform T_m distribution ensures that the selection pressure is driven primarily by binding affinity rather than thermodynamic bias during PCR amplification [27]. This strategy ensures a sufficient template concentration for the subsequent selection round while effectively suppressing the formation of non-specific amplification products or primer dimers.

The application of aptamers in the context of CTC research has gained considerable attention in recent years. Previous studies have primarily utilized aptamers targeting Epithelial Cell Adhesion Molecule (EpCAM) to enrich CTCs [28]. However, the phenomenon of epithelial-mesenchymal transition (EMT) often leads to the downregulation of EpCAM, resulting in the potential loss of highly aggressive CTC subpopulations [29]. Recent literature suggests that CXCR4, a key chemokine receptor associated with tumor metastasis and homing, serves as a more reliable biomarker for identifying CTCs with high metastatic potential, particularly in lung cancer [30]. Unlike traditional antibodies, the aptamers developed in this study offer superior chemical stability and smaller molecular size, which facilitates better penetration into CTC clusters and enables more sensitive in situ detection within microfluidic devices [31].

The integration of these aptamers with the spiral microfluidic chip addresses a critical challenge in liquid biopsy: the rapid and precise identification of viable CTCs from a complex hematologic background. By targeting the CXCR4-positive subpopulation, our platform not only provides a tool for CTC enumeration but also offers a potential means to monitor the dynamic process of cancer recurrence and metastasis. Future efforts will focus on validating this "isolation-identification" workflow in larger clinical cohorts to evaluate its prognostic value in postoperative lung cancer patients.

Declarations

Author Contribution

Shuang Peng performed the experiments, including aptamer screening, microfluidic platform optimization, CTC isolation, and data analysis, and wrote the main manuscript text. Cailian Wang conceived and supervised the study, provided guidance on experimental design and data interpretation, and revised the manuscript. Cailian Wang revised the manuscript critically for important intellectual content and secured funding and resources for the project.

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Figures

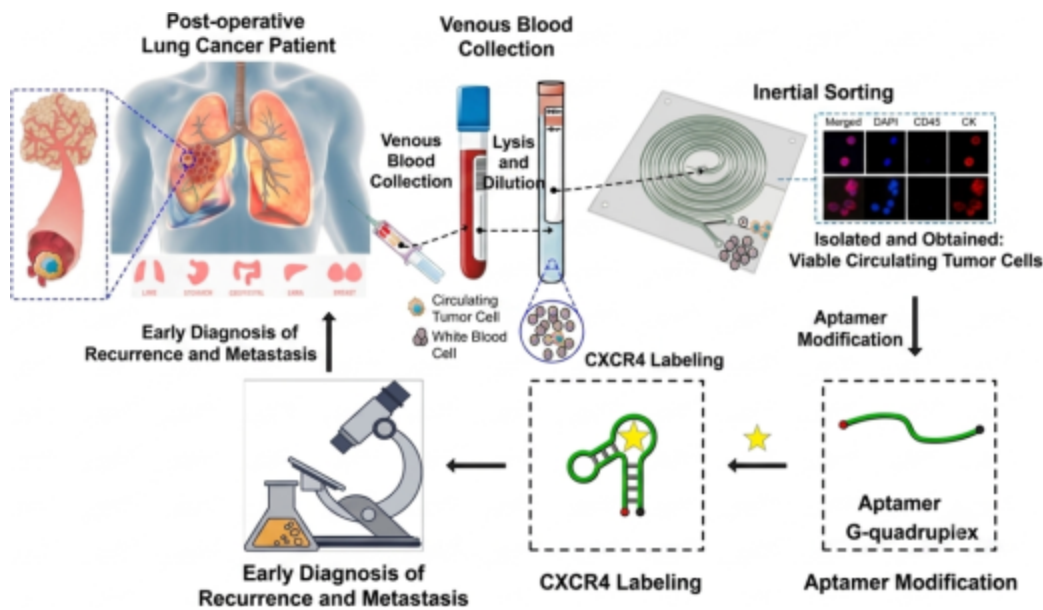


Figure 1

Schematic overview of the integrated strategy for CXCR4+ CTC isolation, molecular characterization, and clinical risk prediction. CXCR4+ circulating tumor cells (CTCs) drive postoperative recurrence via the CXCR4/CXCL12 axis. The workflow combines spiral inertial microfluidics, aptamer-based recognition, multi-omics profiling, and AI modeling to enable early warning of lung cancer metastasis.

CXCR4 sequence with His tag:

MEGISIYSDNYTEEMGSGDYDSMKEP
CFREENANFNKIFLPTIYSIIFLTGIVGNG
LVILVMGYQKKLRSMtdKYRLHLSVADL
LFVITLPFWAVDAVANWYFGNFLCKAV
HVIYTVNLYSSVLILAFISLDLYLAIVHAT
NSQRPRKLLAEKVYVGVWIPALLTIP
DFIFANVSEADRYICDRFYPNDLWVV
VFQFQHIMVGLILPGIVILSCYCIISKLSH
SKGHQKRKALKTTVILILAFFACWLPYYI
GISIDSFILLEIIKQGCEFENTVHKWISIT
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ALTSVSRGSSLKILSKGKRGGHSSVST
ESESSSFHSSHHHHHH*

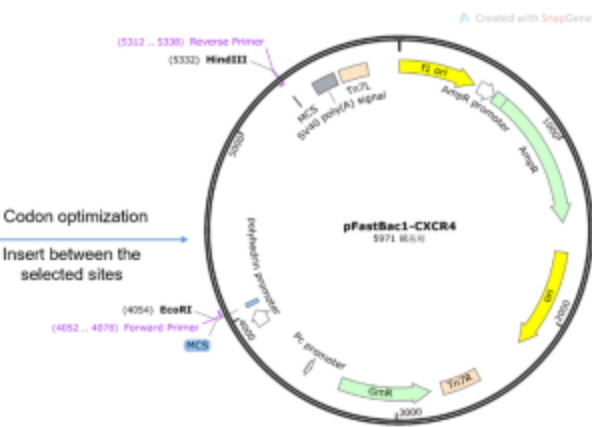


Figure 2

Schematic representation of the recombinant pFastBac-1 plasmid expressing His-tagged CXCR4. The codon-optimized CXCR4 coding sequence fused with a C-terminal His-tag was inserted into the pFastBac-1 vector under the polyhedrin promoter for protein expression in insect cells.

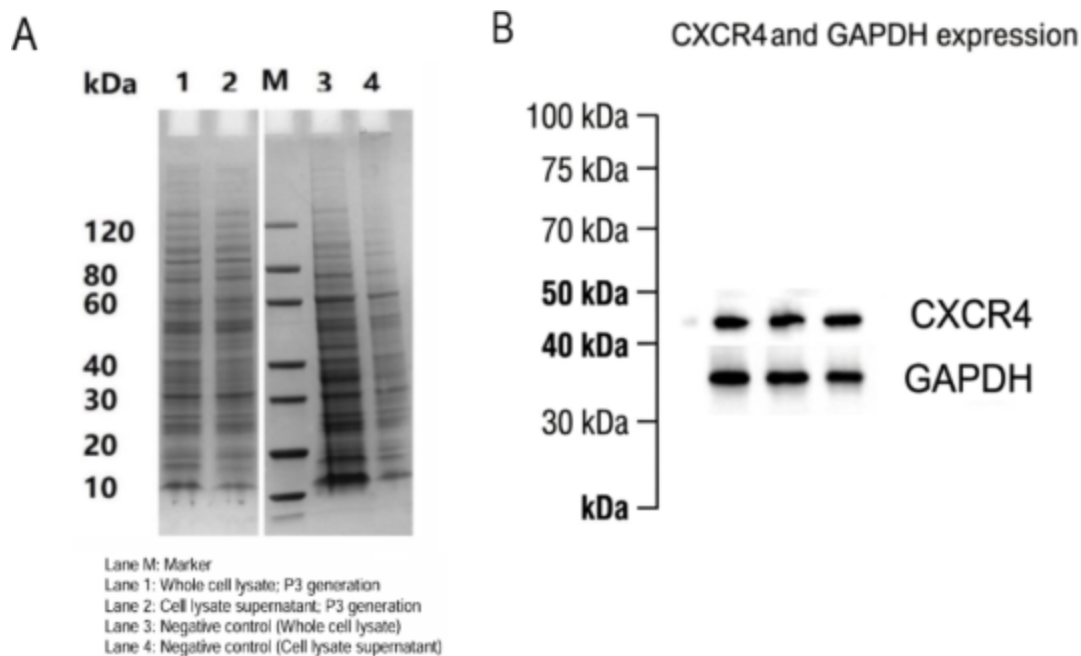


Figure 3

Verification of CXCR4 expression in cells by SDS-PAGE and western blot. A. An additional protein band at approximately 42 kDa was observed in the transfected samples (lanes 1 and 2) but was absent in the negative controls, indicating expression of recombinant CXCR4 protein. B. Membrane was probed with an anti-CXCR4 antibody. GAPDH served as the loading control. The CXCR4-specific band was detected at the expected molecular weight in the sample (P3 generation).

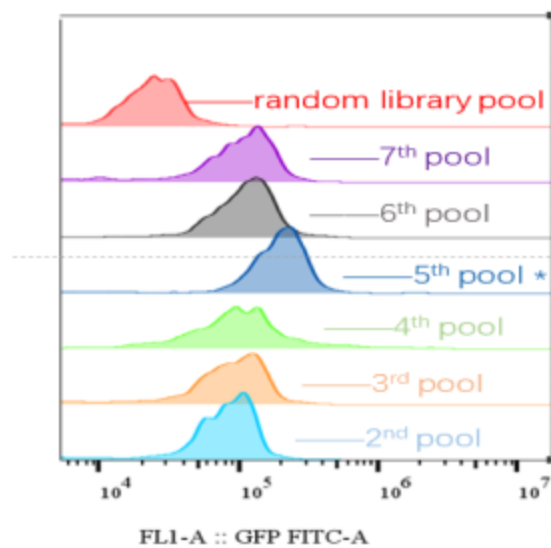


Figure 4

Flow cytometric analysis of the binding affinity of SELEX pools to CXCR4 protein. The PCR-amplified pools from each round (R1–R7) were labeled with 6-FAM via fluorescent primers and incubated with recombinant CXCR4 protein. The fluorescence intensity of each pool was measured by flow cytometry. Round 5 showed the highest binding signal, indicating optimal enrichment of CXCR4-specific aptamers.

