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博士后学位论文

肿瘤细胞与蛋白标志物的核酸适体筛选研究

Selection of aptamers against tumor cells and protein marker

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摘 要

研究报告的第一部分主要以人乳腺癌细胞系MCF-7为靶标，筛选出乳腺癌细胞的核酸适体。乳腺癌是女性常见的癌症之一。然而，乳腺癌的生物标记物却很罕见。既然非正常的膜蛋白是疾病诊断、治疗和预后的理想生物标记物，那么可以利用具有分子识别特性的单链寡聚核苷酸分子（核酸适体）依据细胞表面的差异区分正常细胞和肿瘤细胞并引导发现新的生物标记物。本研究的目标是筛选出人乳腺癌细胞MCF-7的核酸适体，为发现乳腺癌的有效生物标记物奠定基础。因此，本研究采用基于细胞的指数富集配体系统进化技术即Cell-SELEX技术，以人乳腺癌细胞MCF-7为靶标筛选其对应的核酸适体，以人乳腺上皮细胞MCF-10A为对照细胞。在筛选过程中利用流式细胞术监测与乳腺癌细胞MCF-7结合的核酸适体库富集情况。实验所获得的富集的核酸适体库被克隆、测序。经过序列比对后，利用DNA合成仪将具有代表性的高重复性的序列进行化学合成并标记上FAM，随后通过流式细胞术测定各条序列与人乳腺癌细胞MCF-7的结合能力，同时将人乳腺上皮细胞MCF-10A、人乳腺癌细胞MDA-MB-231和MDA-MB-453作为对照细胞系，从而判断各条序列与细胞结合的选择性。实验结果表明，S1序列与人乳腺癌细胞MCF-7结合能力较强，而与乳腺上皮细胞MCF-10A及其它乳腺癌细胞结合力较弱，S1序列与人乳腺癌细胞MCF-7的平衡解离常数Kd为29.9 ± 6.0 nM，因此S1序列为人乳腺癌细胞MCF-7的核酸适体。核酸适体S1可能在乳腺癌生物标记物的发现及乳腺癌早期诊断等方面发挥潜能。

研究报告的第二部分主要阐述了利用琼脂糖液滴微流控技术单分子乳液聚合酶链式反应(PCR)，创建了从复杂单链DNA寡核苷酸库中高效筛选核酸适体的新方法。在传统的指数富集配体系统进化(SELEX)寻找靶标的核酸适体过程中，首先将富集的单链DNA寡核苷酸库进行大量的克隆、测序，接着利用生物信息方法将获得的上百条核酸适体候选序列进行分析、序列比对，然后将挑选出的候选核酸适体分别进行化学合成，再测定各条候选核酸适体与靶标的结合能力，最终获得与靶标结合能力强、选择性高的核酸适体。传统的指数富集配体系统进化筛选靶标的核酸适体的整个过程耗时、费力、低效、高贵。为了解决这些问题，本研究采用了基于琼脂糖液滴微流控技术的高效单分子乳液聚合酶链式反应，建立了高效筛选核酸适体的新方法

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。利用传统SELEX技术筛选出以癌症生物标记物Shp2蛋白为靶标的预富集的单链DNA寡核苷酸库，然后将预富集库按照泊松分布稀释并包裹于独立的均一的琼脂糖液滴中，通过在微流控芯片上的液滴PCR，产生一定比例的包裹单一DNA序列的琼脂糖微球。再将含有DNA序列的琼脂糖微球在显微镜下用毛细管分别挑出，并分别进行PCR扩增放大、单链化，最后利用高通量流式细胞术测定获得的扩增放大的各条单链DNA序列与靶标Shp2蛋白的结合能力。具有高结合能力和低平衡解离常数Kd的DNA序列作为靶标Shp2蛋白的核酸适体，并且获得的核酸适体可以直接应用于后续研究。本研究获得了可以选择性识别Shp2蛋白的核酸适体，核酸适体与Shp2蛋白结合的平衡解离常数Kd为24.9 nM。与传统SELEX的测序-化学合成-结合能力测试的流程相比，本研究建立的方法避免了大量的DNA克隆、测序，避免了昂贵、费时的大量候选DNA序列的合成过程。琼脂糖液滴微流控方法可以应用于分子进化方法，此方法具有高效、廉价等优点，可以广泛应用于分子进化技术，例如mRNA展示，噬菌体展示等。

关键词：核酸适体；乳腺癌细胞MCF-7；指数富集配体系统进化；琼脂糖液滴微流控技术；单分子乳液聚合酶链反应；Shp2蛋白；分子进化

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Abstract

In the first section, aptamer against human breast cancer cell line MCF-7 was selected in vitro. Breast cancer is the most common female cancer. However, the effective specific biomarkers for breast cancer are still scarce. Since abnormal membrane proteins serve as ideal biomarkers for disease diagnoses, therapeutics and prognosis, single-stranded oligonucleotide molecules (aptamer) with molecular recognition properties can be used as efficient tools to sort cells based on differences in cell surface architecture between normal and tumor cells, and discover novel biomarkers. The aim of this study is to screen aptamer against human breast cancer cells MCF-7, laying foundation for identification of specific effective biomarkers for breast cancer. Therefore, cell-based Systematic Evolution of Ligands by Exponential enrichment (Cell-SELEX) process was performed to identify aptamers that targeting surface proteins of human breast cancer cells MCF-7, human mammary epithelial cells MCF-10A as control cell line. The process was repeated until the pool was enriched for sequences that specifically recognizing MCF-7 cells which monitored by flow cytometry. Subsequently, the enriched pool was cloned into bacteria, and positive clones were sequenced to obtain individual sequences. Representative sequences were chemically synthesized, labeled with FAM and determined specificity against MCF-7 cells, using MCF-10A, human breast cancer cells MDA-MB-231 and MDA-MB-453 as control cell lines. As a result, one aptamer against MCF-7 cells named S1 with high binding affinity and equilibrium dissociation constant (Kd) value of 29.9 ± 6.0 nM was identified. Aptamer S1 may be useful for discovery of biomarkers and early diagnosis of breast cancer.

In the second section, we have developed a novel method for efficiently screening affinity ligands (aptamers) from a complex single stranded DNA (ssDNA) library by employing single-molecule emulsion polymerase chain reaction (PCR) based

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on the agarose droplet microfluidic technology. In a typical SELEX process, the enriched library is sequenced first, and tens to hundreds of aptamer candidates are analyzed via a bioinformatic approach. Possible candidates are then chemically synthesized, and their binding affinities are measured individually. Such a process is time-consuming, labor-intensive, inefficient, and expensive. To address these problems, we have developed a highly efficient single-molecule approach for aptamer screening using our agarose droplet microfluidic technology. Statistically diluted ssDNA of the pre-enriched library evolved through conventional SELEX against cancer biomarker Shp2 protein was encapsulated into individual uniform agarose droplets for droplet PCR to generate clonal agarose beads. The binding capacity of amplified ssDNA from each clonal bead was then screened via high throughput fluorescence cytometry. DNA clones with high binding capacity and low Kd were chosen as the aptamer and can be directly used for downstream biomedical applications. We have identified an ssDNA aptamer that selectively recognizes Shp2 with a Kd of 24.9 nM. Compared to a conventional sequencing-chemical-synthesis-screening work flow, our approach avoids large-scale DNA sequencing and expensive, time-consuming DNA synthesis of large populations of DNA candidates. The agarose droplet microfluidic approach is thus highly efficient and cost-effective for molecular evolution approaches and will find wide application in molecular evolution technologies, including mRNA display, phage display, and so on.

Keywords: aptamerbreast cancer cells MCF-7Systematic Evolution of Ligands by Exponential Enrichment (SELEX) agarose droplet microfluidic technology single-molecule emulsion polymerase chain reaction (PCR) Shp2 proteinmolecular evolution

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