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A cell-based single-stranded DNA aptamer specifically targets gastric cancer

Xiaojiao Zhang[a,c,d,1], Jun Zhang[a,c,1], Yanyun Ma[c], Xiaoyu Pei[a], Qingmei Liu[c], Bin Lu[a], Li Jin[c], Jiucun Wang[c], Jie Liu[a,b,*]

[a] Department of Digestive Diseases, Huashan Hospital, Fudan University, 12 Middle Wulumuqi Road, Shanghai, China
[b] Institutes of Biomedical Sciences and Department of Immunology of Shanghai Medical School, Fudan University, Shanghai, China
[c] Ministry of Education (MOE) Key Laboratory of Contemporary Anthropology and State Key Laboratory of Genetic Engineering, School of Life Sciences and Institutes of Biomedical Sciences, Fudan University, Shanghai, China
[d] Department of Chemistry, Fudan University, Shanghai, China

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ABSTRACT

Gastric cancer is one of the most prevailing cancers with high morbidity and mortality. Limitations in the current diagnosis and therapy, specially lacking of specific molecular therapeutic targets, ask for the development of new strategies. Aptamer, a newly developed adaptive molecule, could be used in clinical detection and therapy because of its high affinity and specificity. As no aptamer has ever been developed in preventing gastric cancer so far, we were the first who cloned such an aptamer specifically targeting gastric cancer. The aptamer was selected by systematic evolution of ligands by exponential enrichment with gastric cancer cell-line HGC-27 as target cell line and immortalized gastric epithelial cell-line GES-1 as control cell line. The affinity and specificity of candidate aptamers were examined by flow cytometry, confocal imagining and aptamer-based histochemistry staining. After 19 cycles of systematic evolution of ligands by exponential enrichment and subsequent cloning and sequencing, an aptamer with the highest affinity and specificity (nominated as AGC03) among candidates was screened out from a random single-stranded DNA pool. Moreover, AGC03 could not only specifically bind to gastric cancer cells (the equilibrium dissociation constant value was 16.49 ± 0.40 nM) in vitro, but also recognize cancer cells in human cancer tissue. Our most important finding is that AGC03 could even be internalized into cells automatically. In conclusion, we obtained a novel aptamer specifically targeting gastric cancer, which is an effective tool for both gastric cancer diagnosis and drug delivery.

Brief description: No aptamer has ever been developed in preventing gastric cancer so far. In this study, a novel aptamer is obtained, which can specifically bind to gastric cancer cells and cancer tissue paraffin sections, and can even be internalized into cells and bind to the nucleus automatically. Taken together, our results suggest that this novel aptamer can be used not only in early diagnosis (as sensor), but also in developing novel therapeutic strategy (as targeted transporter or targeted effecter itself) for gastric cancer.

1. Introduction

Gastric cancer is one of the common malignant tumors with high morbidity and high mortality (Leung et al., 2008). No effective strategy for early clinical gastric cancer diagnosis is available yet. The majority of gastric cancer patients were diagnosed in the final stage and thus the overall 5-year survival rate is still very low (Zhang and Li, 2009). It is of great value to develop new tools so as to improve the diagnosis and treatment of gastric cancer.

Aptamer, a new class of adaptive molecule, is single-strand DNA or RNA targeting a wide range of metallic ions, polypeptides, proteins or even cells. It is screened out through SELEX (systematic evolution of ligands by exponential enrichment) technique (Kulbachinskiy, 2007; Sefah et al., 2010) and can be considered as nucleic acid antibody, while its sensitivity and specificity is comparable or even superior to conventional antibodies.

The classic example of aptamer application is Pegaptanib (trade name Macugen) (Chakravarthy et al., 2006; Ng et al., 2006). Macugen is an aptamer specifically binding vascular endothelial growth factor (VEGF) and used to treat age-related macular degeneration. It was the first aptamer drug approved by Food and Drug Administration and became a milestone in the field of aptamer development. As a current trend, drugs coupling with targeted aptamers can be specifically transported to the foci and thus can improve their efficacy while reduce their toxicity (Douglas et al., 2012; Farokhzad

* Corresponding author. Tel.: +86 21 52888236; fax: +86 21 52888236.
E-mail address: jieliu@fudan.edu.cn (J. Liu).
[1] These authors contributed equally to this work.

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et al., 2006; Huang et al., 2009). A recent article on Science Translational Medicine reported such a targeted therapy for HIV (Neff et al., 2011; Zhou et al., 2008), which confirmed the feasibility of utilizing aptamer in the diagnosis and therapy for critical human illnesses.

In cancer research, aptamer technology also turned out to be of great value. Based on it, there have been some breakthroughs in the research of tumor-specific aptamer screening and targeted therapy. For example, the aptamers targeting Glioblastoma (Barbas et al., 2010), acute lymphoblastic leukemia (Shangguan et al., 2008) and small cell lung cancer (Chen et al., 2008) had been reported and applied for visualizing or killing cancer cells in vitro or even in vivo. Further investigation on these aptamers may facilitate the development of diagnosis and targeted therapy for tumors, and may also improve the understanding of the molecular mechanisms of cancer genesis.

In this study, we used gastric cancer cell-line HGC-27 as target cell line and immortalized gastric epithelial cell-line GES-1 as control. After several cycles of SELEX, the aptamer pool targeting HGC-27 were screened out from a random ssDNA library. After cloning and sequencing, an aptamer with the highest affinity and specificity was picked out and named as AGC03. It can specifically bind to gastric cancer cells as well as cancer tissue paraffin sections. Our most important finding is that it could be internalized into cells automatically. Taken together, these results indicated that this novel aptamer can be used to develop new tools for gastric cancer diagnosis and targeted therapy.

2. Materials and methods

2.1. Cell lines and special reagents for cell-SELEX

HGC-27, GES-1 were used for cell-SELEX and the affinity test of candidate aptamers, HGC-27 was used as target (positive cell line) and GES-1 as counter selection (negative cell line). Gastric cancer cell lines MGC-803, SGC-7901 and BGC-823 and lung epithelial cell line Beas-2B, lung cancer cell line H1299, liver cell line HL-7702(L02) and liver cancer cell line HepG2 were used for the specificity test of candidate aptamers. GES-1 was kindly presented from Peking University. All other cell lines were purchased from the cell bank of Chinese Academy of Science.

The unselected library for SELEX was synthesized oligonucleotide DNAs with the following sequence 5′-acgctcggatgccactacag-N_{40}-ctcatggacgtgctggtgac-3′. The sequences of the primers were 5′-acgctcggatgccactacag-3′ and 5′-gtcaccagcacgtccatgag-3. 6-FAM or biotin was labeled at the 5′ end of the primer. The SELEX washing buffer consisted of 4.5 g/l glucose and 5 mM $MgCl_2$ dissolved in PBS buffer (Decent Biotech). The SELEX binding buffer consisted of 4.5 g/l glucose, 5 mM $MgCl_2$, 2 mg/ml BSA and 0.2 mg/ml yeast tRNA dissolved in PBS buffer.

2.2. Cell-SELEX procedure

Cell-SELEX was carried out based on the protocol by Sefah et al. (2010). Approximately 250 pmol (10 nmol for the first round) of ssDNA library/pool was dissolved in 300 μl washing buffer, denatured at 95 °C for 5 min and quickly cooled on ice for 10 min. 5×10^6 GES-1 cells were washed once with washing buffer and suspended in 500 μl binding buffer with an addition of 200 μl FBS (fetal calf serum from New Zealand). The cells were then incubated with the ssDNA pool in an orbital shaker, for 1 h at 4 °C (the incubation time increased with 10 min per round of SELEX, until 2 h). After incubation, the supernatant was absorbed and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant containing the ssDNA sequences was incubated with 5×10^6 HGC-27 cells in an orbital shaker, for 1 h at 4 °C (the incubation time decreased with 5 min per round of SELEX, until 30 min). After that, the cells were washed three times to remove unbound ssDNA sequences. The bound DNA sequences were eluted with 1 ml washing buffer by heating at 95 °C for 5 min and centrifugation. Then the bound sequences were amplified by PCR using FAM- and biotin-labeled primers. The selected sense ssDNA strands were separated from the biotinylated antisense ssDNA by alkaline denaturation (0.2 M NaOH) after affinity purification with streptavidin-coated Sepharose beads (GE Healthcare). After desalination by NAP-5 columns (GE Healthcare), the pool of ssDNA strands was centrifugally dried (DNA 120 SpeedVac Concentrator, Thermo Scientific) for the next round of selection. The entire selection procedure was repeated according to the extent of enrichment. The enriched pool was amplified by PCR using unlabeled primers, the PCR products were cloned into *Escherichia coli* DH5a using pMD19 T-vector (Takara) and the positive clones were sequenced and aligned by MegAlign.

2.3. Monitor the enrichment of aptamer pools with real-time PCR

Real-time PCR reactions were carried out using SYBR Green PCR master mix (10 μl reaction volume) according to the manufacturer's protocol in an Applied Biosystems 7500 sequence detection system (Applied Biosystems). The melting curve was obtained from 7500 software v2.0.1 and was used as the indicator of the enrichment of the aptamer pools.

2.4. Monitor the enrichment of aptamer pools by flow cytometry

27.2 pmol FAM-labeled ssDNA in 40 μl washing buffer was incubated with 3×10^5 target or negative control cells in 50 μl binding buffer with an addition of 10 μl FBS, in an orbital shaker for 30 min at 4 °C. The cells were washed twice with washing buffer and the cells with bound sequences were resuspended in 300 μl binding buffer. The fluorescence intensity was determined with a FACS can cytometer by counting 10,000 events. The washing buffer was used as blank control. The counts were divided into higher fluorescence (M2) and lower fluorescence (M1) parts with the same boundary.

2.5. Determination of aptamer affinity by flow cytometry

Target cells (3×10^5) were incubated with varying concentrations of FAM-labeled aptamer, in an orbital shaker with a 100 μl volume of binding buffer containing 10% FBS for 30 min at 4 °C. The cells were then washed twice with washing buffer, resuspended in 300 μl binding buffer and analyzed by flow cytometry. The washing buffer was used as a blank control. The mean fluorescence intensity of target cells was used to determine the specific binding of the labeled aptamer. The equilibrium dissociation constant (k_d) of the aptamer–cell interaction was obtained by fitting the dependence of intensity of specific binding on the concentration of the aptamers to the equation $Y = B_{max} X/(k_d + X)$, using Sigma Plot (Jandel, San Rafael, CA).

2.6. Confocal imaging

After washed three times with PBS and fixed with 4% paraformaldehyde, target or negative control cells were suspended in 50 μl binding buffer with an addition of 10 μl FBS and incubated with 27.2 pmol FAM-labeled aptamer in 40 μl washing buffer, for 30 min at 4 °C in an orbital shaker. (After the confocal imaging showed that the aptamer could be internalized into the cell automatically, following steps were added). The cells were then washed twice with washing buffer and reacted with 0.5% triton x-100 for 5 min. After two washes with PBS, they were stained with Hoechst (1 mg/ml) for 10 min to show the nucleus. After another