Molecular imaging of HER2-positive tumors via a target-specific 18F-labeled aptamer

Hyun Jeong Kim

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Won Jun Kang

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Hyun Jeong Kim

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This certifies that the Doctoral Dissertation of Hyun Jeong Kim is approved.

Thesis Supervisor : Won Jun Kang

Thesis Committee Member#1 : Tae Joo Jeon

Thesis Committee Member#2 : Won Woo Lee

Thesis Committee Member#3: Ho-Taek Song

Thesis Committee Member#4: Byung Chul Cho

The Graduate School
Yonsei University

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Hyun Jeong Kim

<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

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Department of Medicine
The Graduate School, Yonsei University

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Purpose: Aptamers are single-stranded oligonucleotides that bind to a target molecule with high affinity and specificity. In this study, we undertook to evaluate the target specificity and applicability for in vivo molecular imaging of an aptamer labeled with a radioisotope.

Methods: The human epidermal growth factor receptor 2 (HER2/ErbB2) aptamer was radiolabeled with 18F. HER2-positive tumor cell uptake of aptamer was evaluated in comparison with negative controls by flow cytometry and confocal microscopy. Using 18F-labeled HER2-specific aptamer positron emission tomography (PET), in vivo molecular images of BT474 tumor-bearing mice were taken at each time point.

Results: In flow cytometric analysis, HER2 aptamer showed strong binding to HER2-positive BT474 cells, while binding to

HER2-negative MDA-MB231 cells was quite low. Likewise, in confocal microscopic images, the aptamer was seen to bind to HER2-positive breast cancer cells, with minimal binding to HER2-negative cells. In vivo PET molecular imaging of BT474 tumor-bearing mice showed that uptake of the 18F-labeled HER2 specific aptamer into the tumor was significantly increased. HER2 aptamer was able to preferentially bind to HER2-positive breast cancer cells both in vitro and in vivo, possibly by recognizing HER2 structure on the surface of these cells.

Conclusions: 18F-labeled aptamer enabled appropriate visualization of HER2 expression by human breast cancer cells. The results suggest that radiolabeled HER2 aptamer may have potential applications in determining treatment strategies or in applying targeted therapy against HER2-positive breast cancer cells.

Key words : aptamer; molecular imaging; 18F; PET; HER2

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Ⅰ. INTRODUCTION

Aptamers, from the Latin “aptus”, meaning to fit, and Greek “meros”, meaning region, are single-stranded oligonucleotides ranging from 20-90 base pairs in length. Usually derived using Systematic Evolution of Ligands by Exponential Enrichment (SELEX) methodologies,[1,2] aptamers bind to a target molecule with high affinity and specificity.[3,4] Hence, they are regarded as ideal reagents for detecting and measuring expression of their target molecules. Aptamers have several advantages over antibodies, including their reduced production costs, ease of synthesis, low toxicity, low immunogenicity, and the fact that they do not require an organism for their production.[5] Aptamers are relatively newly developed reagents in the field of theragnosis. Numerous aptamers have been generated against a variety of targets, such as thrombin,[6] nucleolin,[7] prostate-specific membrane antigen (PSMA),[8] tenascin-C (TNC),[9] and viral proteins.[10] In the therapeutic arena, Pegaptanib, a targeted anti-VEGF

(vascular endothelial growth factor) aptamer,[11] was approved by the FDA in 2004 for treatment of macular degeneration. At present, many aptamers are undergoing preclinical and clinical phase evaluation,[12] and more trials with diagnostic and therapeutic oligonucleotides are being carried out. Accordingly, there is a growing demand for feasible methods to evaluate and verify already developed aptamers.

HER2, a well-known oncogene, is amplified or overexpressed in approximately 15-30% of breast cancers.[13,14] It is strongly associated with increased disease recurrence and poor prognosis in several cancers.[15] Two key signaling pathways activated by the HER2 are the Mitogen-activated protein kinases (MAPK) pathway, which stimulates proliferation, and the Phosphatidylinositol 3 kinase – Protein kinase B (PI3K–Akt) pathway, which promotes tumor cell survival.[16] Accordingly, it is regarded as an important therapeutic target in applicable cancers. There are well-known therapeutic monoclonal antibodies targeting HER2, such as trastuzumab and pertuzumab, which are clinically effective. Several DNA/RNA aptamers targeting HER2 have previously been developed via conventional SELEX and cell SELEX.[17-21] The potential pharmacological utility of a HER2 aptamer for tumor inhibition by an endocytosis-mediated mechanism was recently reported.[19]

Molecular imaging is the noninvasive, real-time visualization of biochemical events at the cellular and molecular level within living cells, tissues,

and/or intact subjects.[22] PET is representative molecular imaging modalities. It is a radionuclide molecular imaging technique that enables evaluation of biochemical changes and levels of molecular targets within a living subject. PET has excellent sensitivity and a wide range of applications in the basic research and preclinical arenas, and can easily be applied in the clinical field owing to its negligible pharmacological effects. Tumor targeting by aptamers is an emerging molecular imaging technique. Hicke et al have investigated molecular imaging using aptamers. They conjugated 99mTc to TTA1, an aptamer for the extracellular matrix protein tenascin-C, and obtained γ-camera images of tumors in vivo.[23] Subsequently, PET imaging of tenascin-C with a radiolabeled single-stranded DNA aptamer was reported by Jacobson et al.[24] To the best of our knowledge, PET imaging of a HER2 aptamer has not yet been investigated.

In this study, we conjugated the radioisotope, 18F, to a HER2-specific aptamer in order to validate its target specificity and utility for in vivo molecular imaging.

Ⅱ. MATERIALS AND METHODS

1. Cell culture

The HER2-positive human breast cancer cell line, BT474, was used for in vitro and in vivo experiments. As a negative control for all of the experiments, the human breast cancer cell line MDA-MB231 was used. All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in minimum essential medium supplemented with 10% fetal bovine serum and antibiotics in a humidified incubator at 37°C.

2. Cell lysis and western blotting

To extract cellular proteins, cells were incubated in cell lysis buffer (Invitrogen Life Technologies, Carlsbad, CA, USA) containing protease inhibitors on ice for 30 minutes. Cell lysates were clarified by centrifugation at 14,000 rpm at 4°C for 20 min. Protein concentrations were determined via the Bradford method (Thermo Fisher Scientific, Rockford, IL, USA). For western blot analysis, 30 μg of protein extract from each sample was electrophoresed on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were probed with HER2 antibody (Abcam, Cambridge, MA, USA), and ß-actin antibody was used as a loading control. Signals were developed using an ECL chemiluminescence substrate kit (Advansta, Menlo Park, CA, USA).

3. Annealing, conjugation, and radiolabeling reaction

HER2 aptamers were purchased from Aptamer Sciences Inc (Apsci, Pohang, Republic of Korea) (#SH-1194-35[25]) and Bioneer (Bioneer, Daejeon, Republic of Korea) (2-2(t)[19]). The aptamers were dissolved in dH2O to a final concentration of 2 μg/μL. Aptamers were heated and cooled to permit proper folding of their structures according to the manufacturer's instructions. Fluorescence-labeled single stranded DNA was purchased from Bioneer (Bioneer, Daejeon, Republic of Korea). Oligonucleotides were labeled with 18F using click chemistry.[26,27] Oligonucleotides were annealed at their calculated melting temperatures (Tm's) and allowed to slowly cool to room temperature. The secondary folding structure and Tm of each oligonucleotide was calculated on the basis of its lowest free energy structure, using Oligocalc web servers.[28,29]

4. Radiolabelling of 18F-Fluorobenzoyl Aptamer

18F-FB aptamer was synthesized according to previously reported procedures [24] with some modifications. N-succinimidyl 4-18F-fluorobenzoate (18F-SFB) was synthesized via three-step, one-pot method[30-32] by synthesizer (Tracerlab FXFN, GE Healthcare, Milwaukee, WI, USA). Purified 18F-SFB (0.78-1.48 GBq) was reconstituted in dimethylformamide. Amine-terminated HER2 aptamer (5-10 nmol in PBS, pH 8.5) was added to the 18F-SFB residue, and the mixture was stirred for 30 minutes at 37℃. Subsequently, the mixture was purified by using reverse phase high performance liquid chromatography (HPLC) with a semi-preparative C18 column (Xbridge OST C18 10×50 mm,

gradient acetonitrile/0.1M TEAA 5:95-95:5 over 20 minutes) at a flow rate of 5 mL/min, equipped with a UV (254 nm) detector and a radioactivity detector. The HPLC fraction containing 18F-FB aptamer was diluted with water and passing through a C18 Sep-Pak® cartridge (Waters Corporation, Milford, MA). The labeled aptamer was then eluted with 500μL of ethanol and formulated in normal saline for in vivo experiments.

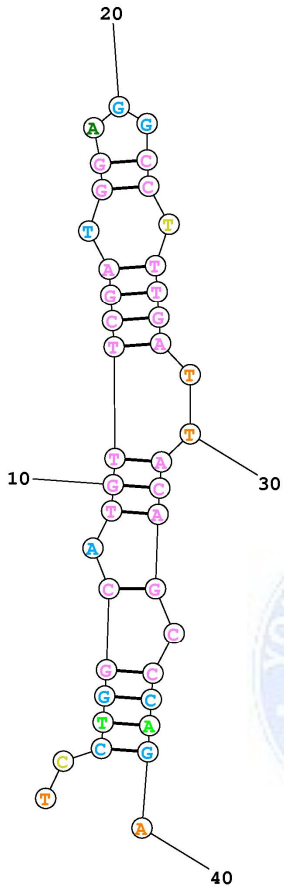

Figure. 1. The predicted secondary structure of aptamer SH-1194 based on a lowest free energy model.

5. Confocal microscopy

BT474 or MDA-MB231 cells were seeded onto coverslips and cultured overnight. The cells, at > 80% confluence, were carefully washed and then incubated with the fluor-labeled aptamer at a final concentration of 250 nM. After incubation, cells were carefully washed and mounted on slides on mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). The fluorescent signal was detected via LSM700 confocal microscopy (Zeiss, Jena, Germany). Excitation wavelength and emission filters were as follows: fluorescein isothiocyanate (FITC), 488 nm laser line excitation, emission BP490-555; and Texas red, 639 nm laser line excitation, emission LP640 filter.

6. Flow cytometry

The specificity of the HER2 aptamer was evaluated with a fluorescence-activated cell sorting (FACS) LSR II Flow Cytometer System (BD Biosciences, San Jose, CA, USA). BT474 and MDA-MB231 cancer cells were seeded onto Petri dishes in complete medium and grown to 80% confluence. Cells were treated with trypsin, washed, and incubated at 4°C for 30 min with the fluor-labeled HER2-specific aptamer or antibody (Biolegend, San Diego, CA, USA) as a control in saline containing 1% fetal bovine serum. After washing, bound aptamer molecules were detected, and cells were analyzed using a fluorescence-activated cell sorter.

7. In vivo experiment

Four-week-old female Balb/c nude mice were implanted subcutaneously with 17β-estradiol pellets (Innovative Research of America, Sarasota, FL, USA) at the lateral side of the neck, with dosage release rates sufficient to permit estrogen-dependent tumorigenesis. After a few days, mice were inoculated intradermally with 7×10^6 BT474 human breast cancer cells per mouse. Tumors were allowed to develop for 3 weeks before imaging studies were performed. Tumor growth was monitored by caliper measurement.

8. 18F-aptamer PET imaging

We intravenously injected radiopharmaceutical-labeled aptamers into mice and performed PET imaging using a Siemens Inveon PET (Siemens Medical Solutions USA Inc., Knoxville, TN, USA). The injected dose was 13.7 ± 1.1 MBq (370 ± 30 μCi). A dynamic PET study was performed for 30 minutes with the following protocol: ten 1 min images and four 5 min images. Two static studies were then performed, for 10 minutes each at 60, 90 and 120 minutes after injection. Semi-quantification of PET signal was performed using AMIDE software (SourceForge, New York, NY, USA). Images are presented here using a false-color scale that is proportional to tissue concentration (%ID/g) of positron-labeled probe. Red represents the highest, with yellow, green, and blue corresponding to progressively lower concentrations.

Ⅲ. RESULTS

1. Verification of HER2 expression in, and aptamer affinity for, target tumor cells

Western blot and flow cytometry assays were performed to investigate the expression of HER2 in BT474 breast cancer cells. Western blot analysis confirmed overexpression of HER2 in BT474 as well as in SKBR3 cells, which are known to overexpress HER2 due to gene amplification.[33] The negative control cell line, MDA-MB231, showed no signal in the corresponding location (Figure 2).

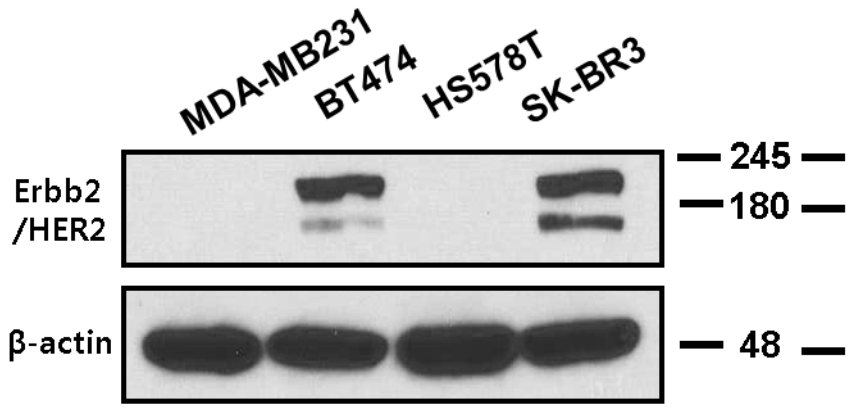

Figure 2. HER2 expression characteristics as determined by western blotting of human breast cancer cell lines. The BT474 cell line highly expressed HER2, whereas MDA-MB231 cells had no detectable HER2 expression. Beta-actin was used as a loading control (lower panel).

As seen in Figure 3, HER2 antibody showed highly specific binding to the HER2-positive BT474 cancer cells in flow cytometry analysis. Compared to the antibody, HER2 aptamer (SH-1194) also had relatively strong binding to BT474 cells, while binding to MDA-MB231 cells was quite low. In addition, a random DNA oligonucleotide showed no significant binding preference for either cell line. These results suggested that the HER2 aptamer preferentially bound to HER2-positive breast cancer cells, possibly by recognizing the HER2 structure on the surface of these cells.

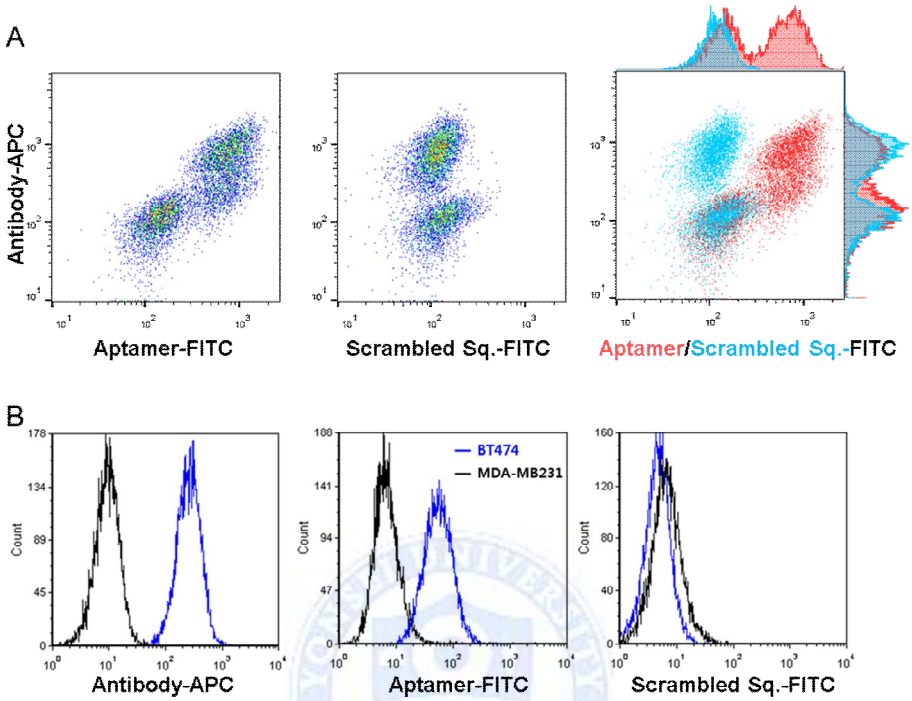

Figure 3. Flow cytometry analysis of breast cancer cell lines using HER2 antibody and aptamer. (A) Dot plots representing the fluorescence signals for BT474 (HER2-positive cells), and MDA-MB231 (HER2-negative cells) from monoclonal antibody and SH-1194 aptamer (red) or scrambled random sequence (blue). (B) Flow cytometric histogram of two cells using antibody, aptamer, and negative control.

2. Confocal microscopic analysis

The binding of the selected aptamers to cells was further assessed by confocal microscopy (Figure 4). BT474 HER2-positive breast cancer cells were treated with conjugated aptamer. FITC-labeled SH-1194 aptamer was readily visualized on the cell surface, indicating occupation of the HER2 structure on the surface of these cells. The presence of the HER2 aptamer (green) was visualized along cell membrane. In contrast, MDA-MB231 cells, the negative control, showed no significant fluorescence of the aptamer, which indicates absence of HER2 target. The SH-1194 aptamer was found to be able to bind HER2-positive breast cancer cells, with minimal binding to HER2-negative cells.

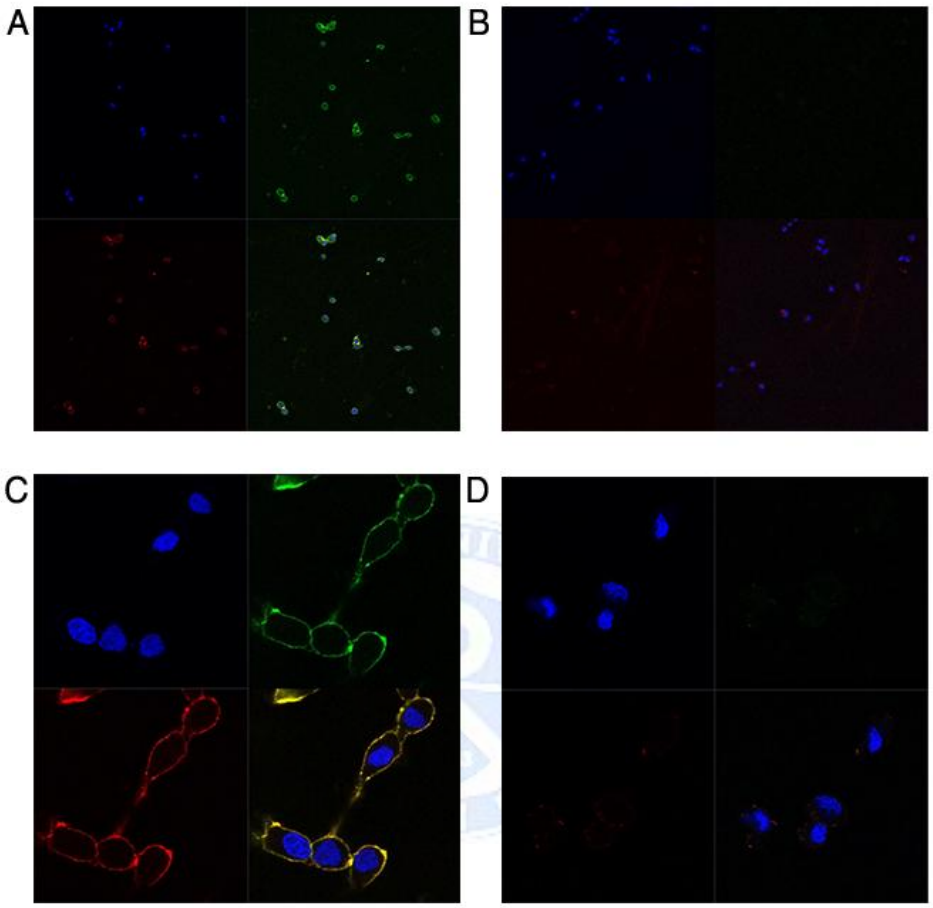

Figure 4. Confocal microscopic images of selected aptamers to HER2 postivie cell.

(A, B) Low- and (C, D) high-magnification images from confocal laser scanning microscopy. (A, C) BT474, HER2-positive, breast cancer cells were transduced with fluor-labeled aptamer. (B, D) MDA-MB231 cancer cells with treated with aptamer. (Labeling, blue: DAPI; green: FITC-aptamer)

3. In vivo molecular imaging

Using animal micro PET, in vivo molecular images of BT474 tumor-bearing mice were taken at each time point. As seen in Figure 5, 18F-labeled HER2 specific aptamer PET showed significantly increased uptake into the left axillary tumor. In images taken at 120 minutes, the tumor was clearly labeled by SH-1194 aptamer in axial and coronal images. Physiologic uptake into the bowel and bladder was predominant, reflecting the two major clearance pathways of radiopharmaceuticals. Figure 6 shows representative images of 18F-labeled HER2 aptamer PET in HER2 positive and negative tumor-bearing mice. HER2 overexpressing BT474 tumor shows more increased uptake compared to the HER2 negative MB-MDA231 tumor. For semi-quantitative analysis, total activity of (nCi) each VOI (voxel- or volume-of-interest) was calculated. Comparison of T/M (tumor/muscle) uptake ratio between BT474 and MDA-MB231 cell line demonstrates higher imaging contrast and T/M ratio in HER2 overexpressing BT474 tumor (Figure 7).

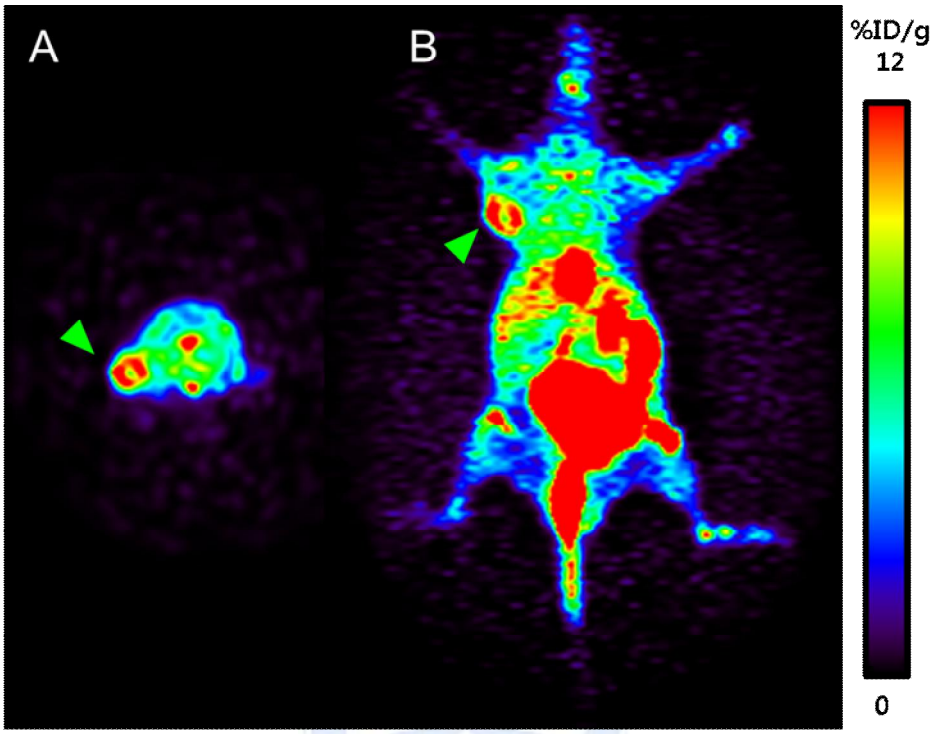

Figure 5. Representative images of in vivo 18F-labeled HER2 aptamer PET in a BT474 tumor-bearing mouse. Focal increased uptake in the left axillary tumor is seen in the axial (A) and coronal PET images (B).

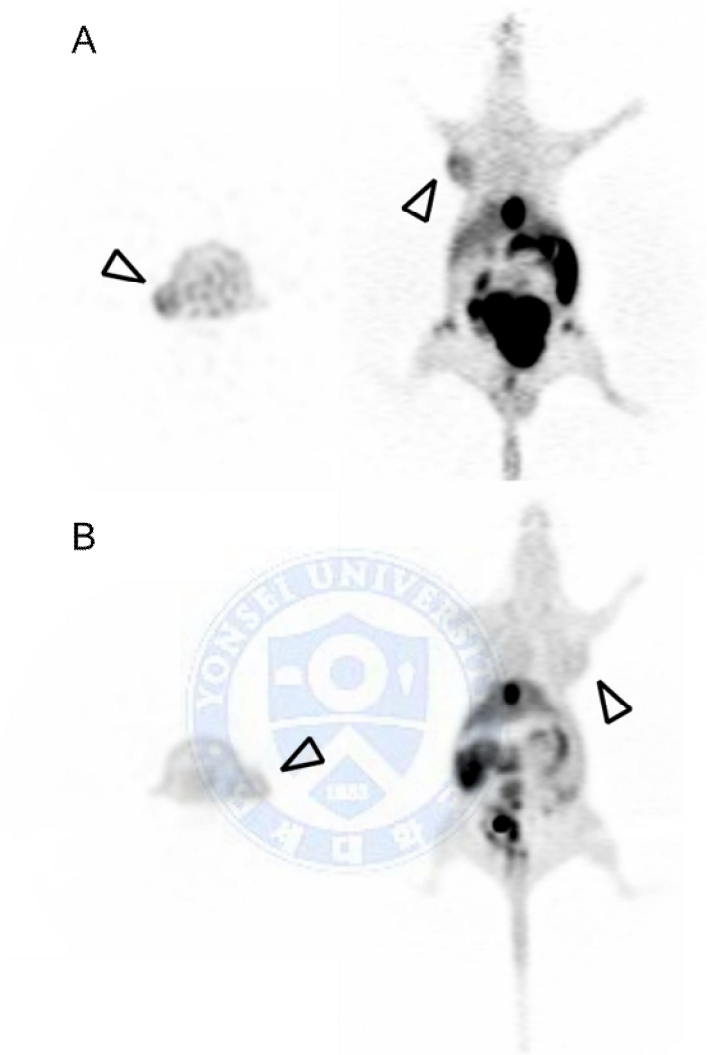

Figure 6. Representative images of in vivo 18F-labeled HER2 aptamer PET in HER2 positive and negative tumor-bearing mice.

(A) HER2 overexpressing BT474 tumor (Lt axilla) shows more increased uptake compared to the (B) HER2 negative MB-MDA231 tumor (Rt axilla).

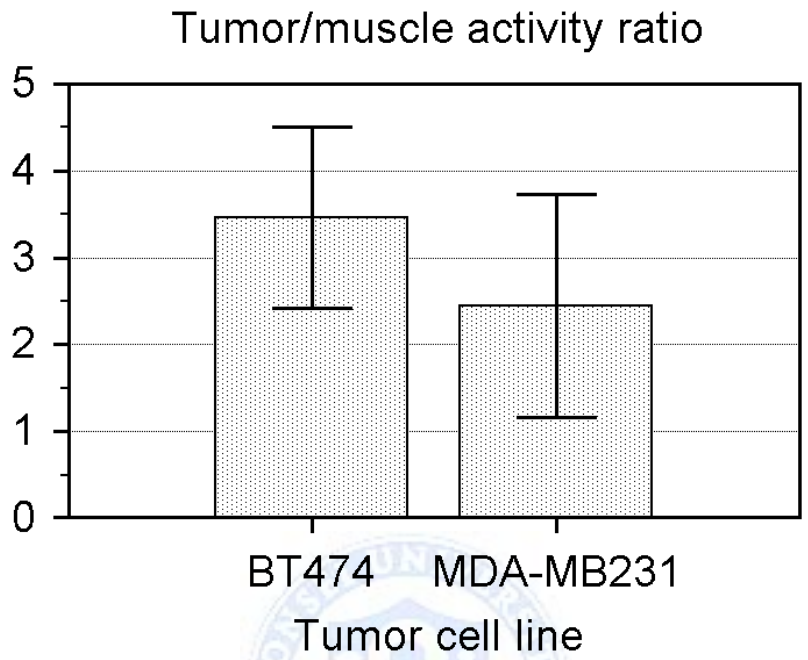

Figure 7. Tumor/muscle ratios ofn 18F-labeled HER2 aptamer PET. Comparison of tumor uptake ratio between BT474 and MDA-MB231 cell line represented significantly higher BT474 tumor uptake ratio. (n=3/group)

Ⅳ. DISCUSSION

The primary objective of this study was to investigate tumor-specific PET imaging using radiolabeled aptamers. In this study, SH-1194, a HER2-targeted DNA aptamer, was successfully PET imaged in vivo. Since its first description in 1990,[1] numerous SELEX methods have been investigated and many novel aptamers have been developed. Among them, we chose several HER2-targeted DNA aptamers as candidates. Previous studies have demonstrated the utility of HER2 aptamers in inhibiting tumorigenic growth, both by themselves[19] and by delivering cytotoxic drugs.[18] There are a few reports on the use of aptamers to image HER2-targeted cancer. Recently, an attempt to apply molecular imaging using Tc-99m labeled aptamer was reported describing biodistribution data without images.[34] This is the first report of HER2-targeted PET imaging. PET images of BT474 tumor-bearing mice showed reliable tumor-to-background ratios, which implies that the aptamer recognized the HER2 target in vivo.

In this study, flow cytometry and confocal microscopic analysis were performed to verify target-binding affinity of aptamers, There are many aptamers available for any given specific target. To determine which aptamer is appropriate for any particular study, assessment of affinity is required. SH-1194, a modified DNA aptamer designed to target HER2, showed superior performance in in vitro experiments, and thus we adopted this aptamer for PET

imaging.

Many therapeutic aptamers are being established in clinical trials for pharmaceutical safety and efficacy.[35-38] Among others, AS1411, a DNA aptamer targeting nucleolin, shows anti-tumor efficacy against renal cell carcinoma, and has completed a phase 2 trial.[39] Other aptamers, like ARC1779, targeting activated von Willebrand Factor (vWF), have verified efficacy against purpura and thrombotic thrombocytopenic disease. In addition, several RNA aptamer conjugates targeting PSMA[40] or designed to facilitate PSMA-targeted drug delivery[41] have reinvigorated research in the field. Likewise, due to tremendous translational potential for aptamers in clinical applications, there are many aptamers and aptamer-based drugs being evaluated for the treatment of a variety of disorders, involving those of coagulation, cancer, and inflammation.

Modification of magnetic nanoparticles or fluorescent agents with aptamers might provide good contrast agents for targeted fluorescence imaging and magnetic resonance imaging (MRI). Several in vivo MRI studies on tumor-bearing mice demonstrated efficient targeting to tumors.[42,43] However, since metabolic changes commonly occur before anatomical changes, PET has a clear diagnostic advantage over anatomical techniques such as computed tomography (CT) and MRI. In addition to its clinical utility, PET has a wide range of applications in the basic research and preclinical arenas. For example, PET can be used to evaluate novel radiopharmaceuticals, effectiveness of new

therapies, and biodistribution of pharmaceuticals. PET has key strengths in its depth of penetration, excellent sensitivity, quantitative data, and translatability from the pre-clinical to clinical stage.[22] However, there have been only a few studies that have attempted to establish PET imaging of aptamers. Recently, PET imaging of tenascin-C with a radiolabeled single-stranded DNA aptamer was reported.[24] A tenascin-C aptamer, labeled with 18-F or 64-Cu, demonstrated reliable tumor uptake compared to the nonspecific scrambled aptamer. However, comparison with a conventional monoclonal antibody was not validated in that study.

The application of aptamers in cancer therapy is emerging and is being extensively studied at present. Our data suggest its potential for use as a target-specific molecular imaging tool for determining course of treatment. Aptamers or aptamer-based drugs may provide alternative options for the treatment of HER2-positive refractory malignancy, since aptamers may use different mechanisms than monoclonal antibodies do to occupy certain target molecules, and not provoke an immune reaction. In addition, because of their highly specific target affinity and applicability, aptamers might be ideal carriers for drugs or toxins. This has been demonstrated by Zhe Liu et al,[18] who showed that a complex of aptamer and doxorubicin could selectively deliver doxorubicin to HER2-positive breast cancer cells, with minimal binding to HER2-negative cells.

There are several limitations inherent in this study. We have not yet determined the binding mechanism of the HER2 aptamer. Further research is needed to establish the molecular biologic mechanisms of aptamers. In murine PET images, a reliable tumor-to-background ratio was observed. Nonetheless, physiologic uptake in the bowel was still predominant. In order to eliminate physiologic uptake, a delayed image with clinically sufficient biologic half-life of a pharmaceutical is mandatory. One solution may be to attach polyethylene glycol polymer chains (PEGylation) to aptamers to increase their stability.

Ⅴ. CONCLUSION

Aptamers represent an emerging class of target-specific molecular imaging tools. In this study, 18F-labeled aptamer permitted appropriate visualization of HER2-expression by BT474 tumors in vivo. These results suggest that radiolabeled HER2 aptamer may have potential applications in determining treatment strategies or in applying targeted therapy against HER2-positive breast cancer cells.

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ABSTRACT(IN KOREAN)

18F-표지 압타머를 이용한 HER2 종양의 표적특이적 분자영상

<지도교수 강원준>

연세대학교 대학원 의학과

김현정

압타머란 단일사슬 구조의 올리고뉴클레오타이드로서 특정 목표물에 높은 친화성과 특이성을 갖고 결합하는 물질이다. 본 연구의 목적은 압타머에 방사성동위원소를 부착하여 특정물질에 특이적인 분자영상을 얻고 그 유용성을 고찰하고자 하였다.

Human epidermal growth factor receptor 2 (HER2/ErbB2)에 대한 압타머를 18F으로 표지하였다. HER2를 과다 발현하는 세포주와 대조군에서 압타머의 친화성을 유세포분석과 공초점레이저현미경를 통해 평가하였다. 마우스를 이용한 생체실험으로, 18F 표지 HER2 압타머를 정맥주사하고 동물 positron emission tomography (PET) 영상을 얻었다.

유세포분석 결과, HER2 압타머는 HER2를 과다 발현하는 BT474 세포에 강한 친화성을 보인 반면, HER2 음성 MDA-MB231 세포는 아주 낮은 친화성을 보였다. 마찬가지로, 공초점레이저현미경 이미지에서 HER2 양성 세포에는 세포 표면에 압타머가 부착된 것이 잘 관찰되었다. 마우스 BT474 종양모델에서 PET 영상을 얻은 결과, 해당 종양 부위에 18F가 부착된 압타머의 신호섭취가 매우 증가되어 있었다. In vitro, in

vivo 실험 모두에서 HER2 압타머는 HER2 양성 유방암 세포에 높은 친화성을 가지고 특이적으로 결합하는 것을 확인하였으며 이는 압타머가 세포 표면 HER2 구조를 인식하는 것에서 기인한다.

18F 표지 압타머는 유방암 세포의 HER2 발현을 잘 영상화 할 수 있다. 이 결과는 방사성동위원소가 표지된 압타머의 진단, 치료 방법 설정 및 효과 예측에 잠재적 활용가능성을 시사한다.

핵심되는 말 : 압타머, 분자영상, 18F, PET, HER2