Biosensors and Bioelectronics 40 (2013) 161–166

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Micro-fluidic chip platform for the characterization of breast cancer cells using aptamer-assisted immunohistochemistry

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ARTICLE INFO

Available online 17 July 2012

Keywords:
Immunohistochemistry
Aptamer
NSABA
Microchip platform
Breast cancer characterization

ABSTRACT

We demonstrated a new QIHC (Quantitative Immunohistochemistry) microfluidic PDMS (Polydimethylsiloxane) platform by the introduction of the aptamer specific to the Fc region of the IgG antibody as a reporting probe. The aptamer was designed and synthesized. Various breast cancer cell lines were prepared as paraffin block slides, which were covered by a microfluidic PDMS platform to form a micro-reaction chamber. Primary antibodies specific to marker proteins (HER2, ER, PR, and ki-67) for breast cancer characterization were loaded in the micro-fluidic chip prior to the introduction of the aptamer. A master mixture of QNASBA (Quantitative Nucleic Acid Sequence Based Amplification) was used to quantify marker proteins by real time amplification of the aptamers. The quantitative results of aptamer amplification were linearly proportional to the concentrations of 4 different primary antibodies. The characterization results of the aptamer-assisted IHC using the microfluidic platform were well-correlated with those of conventional IHC for breast cancer cell lines (SK-BR-3, MCF-7, MDA-MB-231). Objective quantitative evaluations were carried out and compared with conventional results for real clinical samples.

1. Introduction

Nowadays, the characterization of cancer tumors has been focused on essential processes to achieve "personal medical treatment" as a new cancer therapy, which could provide tailored medicine specific to individuals. Several personalized anti-cancer drugs have been developed and commercialized based on specific cancer characterization (van't Veer and Bernards, 2008).

Immunohistochemistry (IHC) has been widely utilized as a well-established molecular diagnostic method of cancer tumors in the clinical field for over 20 years. Particularly, it has been utilized as a powerful clinical tool to offer objective information about a tumor when it is indistinguishable by morphological study, or when it is a borderline tumor according to immunofluorescence methods (Harvey et al., 1999).

IHC employs a signal amplification step involving the introduction of enzyme-labeled secondary antibodies to surpass the early stage immunofluorescence method by single organic fluorophore (Hsieh et al., 2011; Lebeau et al., 2001). Various signal amplification methods have already been developed using chemical and biological methods such as the fluorescent antibody method (Nehme et al., 2011; Wang et al., 2011), the enzyme-labeled antibody method (Lequin, 2005), avidin-biotin-peroxidase complex (ABC) (Morimoto et al., 2009), peroxidase–antiperoxidase complex technique (PAP technique) (Bratthauer, 2010) and immunogold technique (Fernandez-Alacid et al., 2011; Lin et al., 2008). The most widely used method in the clinical field involves the use of an anti-IgG antibody as a secondary reporting probe labeled by a chromophoric enzyme such as HRP (Horseradish Peroxidase), because this enzymatic product can be easily visualized by microscopic investigation (Joshi et al., 2008). However, this conventional and commercial IHC can only give qualitative and eidetic quantitative results using microscopic investigation, and may induce false diagnostic results. Therefore, various quantitative IHC (QIHC) tools have been studied for direct application to personalized cancer therapy since the early 2000s (Dowsett and Dunbier, 2008). The representative methods to quantify the marker concentrations on tumor cells use an image digitizing system that can measure the intensity or pixel number of color induced by fluorophores or chromophoric-enzyme-labeled antibodies (Xing et al., 2007). However, four processes—first antibody binding on target protein, second antibody labeling, second antibody binding on the first antibody, and the reaction of enzyme tagged on secondary antibody for signal amplification—are still required to quantify the concentration of markers on the tumor cells.

These sophisticated processes may increase the detection error, so simplification is required to increase the detection reliability. However, the use of secondary antibody has limited

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http://dx.doi.org/10.1016/j.bios.2012.07.004

the process simplification. New concepts for reporter probes are necessary for QIHC. One of the representative materials is aptamer. Also known as chemical antibodies, they normally consist of tertiary structures of single-stranded DNA or RNA. It is possible for aptamers to compete with biological antibodies because they often have similar affinity for a target under specific SELEX (Systematic Evolution of Ligands by EXponential enrichment) conditions. Among the various advantages of aptamers, such as structure designable for a target, convenient immobilization by well-defined DNA chip technology, and chemically synthesizable materials, we have focused on the fact that aptamers can be amplified simply by well-defined nucleic acid amplification methods. This allows us to simplify the whole QIHC process and increase the reproducibility of the QIHC just by introducing an aptamer as a reporting probe, because aptamers can play both roles of secondary antibodies and signal amplification labels when nucleic acid amplification methods are combined with QIHC. We have introduced an aptamer as a reporting probe and signal amplifier, not as first antibodies specific to markers, because the ability of aptamers to alternate primary monoclonal antibodies is still doubtful, and they are not yet universally accepted as a clinical tool (Jayasena, 1999; Lee et al., 2008). However, an aptamer alternative to anti-IgG could be scientifically valuable as a universal signal amplification tool in the antibody-based biosensor field because the moderate affinity of the aptamer is enough to be utilized as secondary polyclonal antibodies.

It is technologically difficult to combine nucleic acid amplification tools with marker detection by antibodies in clinical samples. This combination of two different methods in one single sample can be realized by the introduction of microfluidic technologies. Microfluidic devices have already been applied to IHC to reduce labor, processing time, and reagent cost (Kim et al., 2011). However, this is the first time antibody reaction and nucleic acid amplification steps have been combined in a single chamber.

In this study, as shown in Fig. 1, we develop an Fc-region-specific aptamer as a reporter probe in QIHC, and employ it to characterize breast cancer tumor cells, which are already clinically categorized by protein markers (HER2, ER, PR, ki-67). The aptamer is simply amplified by Quantified-Nucleic-Acid-Sequence Based Amplification (NASBA), which is an isothermal, RNA-preferred amplification reaction (Min and Baeumner, 2002; Baeumner et al., 2003; Won and Min, 2010; Won et al., 2010). Samples are prepared in the form of a paraffin block slide since most clinical tissue samples are frozen and blocked by paraffin for a long storage. The new aptamer-assisted QIHC (AA-QIHC) microfluidic platform is suitable for paraffin block slides, and is designed, fabricated, and applied to breast tumor samples. These proposed tools are first verified with three different breast cancer cell lines (SK-BR-3, MCF-7, and MDA-MB-231) because it is well-known that the lines have totally different characteristics. The verified aptamer QIHC is also tested and compared with real clinical samples.

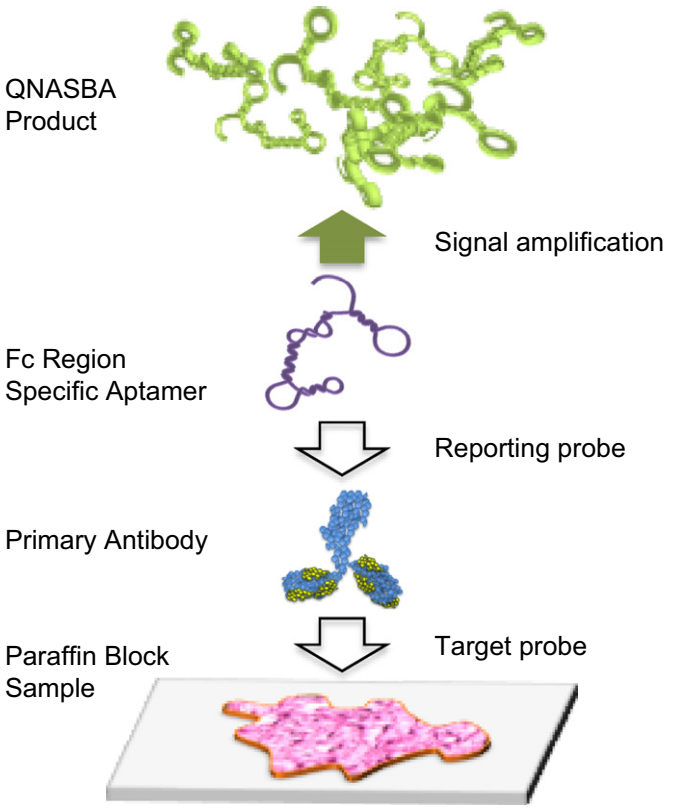

Fig. 1. Schematic illustration of Aptamer-assisted Immunohistochemistry (AA-IHC).

2. Experimental detailis

2.1. Fc-specific aptamer selection

As the aptamer, ssRNA oligonucleotides were used, which contains a central randomized region of 40 nucleotides flanked by two conserved 22-nucleotide and 27-nucleotide regions in each end (5′-GGGAGAGCGGAAGCGUGCUGGG–N_{40}–AUAACCCAGAGGUCGAUGGAUCCGGGG-3′).

A solution containing synthesized random ssRNA at a concentration of 10^7 base sequences/mL was added to SELEX buffer (50 mM Tris–HCl (pH 7.4), 5 mM KCl, 100 mM NaCl, 1 mM $MgCl_2$, 0.1%NaN_3 (Sigma Aldrich; USA)), heated at 80 °C for 10 min, and allowed to stand on ice for 10 min.

The detailed SELEX procedures are described in the reference (Kim, 2010). 5 candidates were selected, and a single sequence ($K_d \approx 3$ nM) was chosen after protein chip analysis to compare signal density.

2.2. Aptamer amplification

The aptamer was amplified using the isothermal QNASBA method. The primers, 5′-GGGAGAGCGGAAGCGTGCTGGG-3′ and 5′-AATTCTAATACGACTCACTATAGGG-CCGGATCCATCGACCTC-3′, were contained in a NASBA reaction solution. NASBA reaction was performed at 41 °C for 60 min. The NASBA amplification was carried out in each chamber. To measure the NASBA product, SYBR® Green II (Invitrogen; USA) was added to the NASBA mixture. All experiments were quintuplicated.

2.3. Sample preparation

Antibody layers on a solid surface were required to verify the capability of the aptamer as a reporting probe. 4 monoclonal antibodies specific to the 4 different markers (HER2, ki-67; Dako, Denmark/ER, PR; Ventana, Tucson, AZ) were loaded and kept in each PDMS micro-chamber (width × height × length = 800 μm × 50 μm × 5 mm) with different concentrations (0.1, 1, 10, 100, and 1000 ng/μL) for 30 min. After non-specific fouling of each primary antibody on the PDMS surface, unbound antibodies were washed out by PBS buffer (1X) at a speed of 100 μl/min, and the PDMS

surface was treated by BSA to prevent non-specific binding of primary antibodies.

Paraffin block slides containing breast cancer cell lines with well-established characterization were prepared to perform IHC tests. SK-BR-3, MCF-7, and MDA-MB-231 cells were obtained from Lonza, Ltd (USA). These cells were cultivated in RPMI-1640 (PAA; Austria) supplemented with 10% FBS (GIBCO; USA), 1% penicillin–streptomycin (GIBCO; USA), and 1% GlutaMAX (GIBCO; USA) at 37 °C with 5% CO_2. Adherent cells were harvested by trypsinization. For QIHC analysis, the harvested cells were centrifuged, fixed in 10% neutral buffered formalin (BBC Biochemical; USA), suspended in agar, and embedded in paraffin. Paraffin blocks of cells were sliced with 5 μm thickness using an HM 325Rotary Microtome (Thermo Scientific; USA). The sections were baked onto positively charged slides and allowed to dry for 1 h at 60 °C.

2.4. Microfluidic platform fabrication

The PDMS platform was fabricated by soft lithography using SU-8 2025 (Microchem Corp.; USA) patterns on silicon wafer as a template. The PDMS was prepared using a SYLGARD® 184 SILICONE ELASTOMER KIT (DOW CORNING, USA). As shown in Fig. 2 (also see Figure S1), the microfluidic platform consisted of 8 micro-reaction chambers (1st to 3rd chambers for quantitation calibration of aptamer amplification, 4th chamber for negative control, and 5th to 8th chambers for sample characterization with HER2, ER, PR, and ki-67). Each micro-reaction chamber was 800 μm wide and 5 mm long, and used to apply on the paraffin block slide samples (Recent results provided by the National Cancer Center of Republic of Korea have shown that $>93\%$ of biopsy breast tissues had tumor sizes >4 mm in diameter (Kim et al., 2010)). For simultaneous monitoring of fluorescence signals from all 8 chambers, 8 output channels (with widths of 100 μm and spacing of 100 μm) were gathered into the detection window, which was 1.5 mm wide and 1.4 mm long. Through the detection window, fluorescence signal images of the 8 different micro-channels were simply captured using the JuLI™ smart fluorescent cell analyzer (NanoEnTek; Republic of Korea) for 1 h.

2.5. Aptamer-Assisted Quantitative ImmunoHistrochemistry (AA-QIHC)

To achieve objective QIHC results using the aptamer, the microfluidic PDMS platform was placed on the paraffin block samples. First, 100 ng/μl of the primary antibodies (HER2, ER, PR, ki-67) were loaded into each of the 5th–8th micro-chambers and kept for 30 min. A washing procedure was performed with 100 μl/min of PBS buffer (1X) for 5 min. Aptamer (10^5 copy/ml) in SELEX buffer was filled into the 5th–8th micro-chambers, whereas aptamers (10^4, 10^3, 10^2, 0 copies/5 μl) for calibration signals were filled into the 1st–4th microchambers. The master mixture for QNASBA was filled into the 1st–8th micro-chambers and amplified for 60 min at 41 °C after removing unbound aptamers in the 5th–8th micro-chambers.

3. Results and discussion

3.1. Quantitation of markers by aptamer

To verify the role of the aptamer designed in this study as a quantitative signal amplifier, the aptamer was amplified by QNASBA method. The real time signal of QNASBA was defined as a time of threshold (T_T) similar to the cycle of threshold (C_T) in real time PCR (Figure S2). As shown in Fig. 3(a), the T_T values were

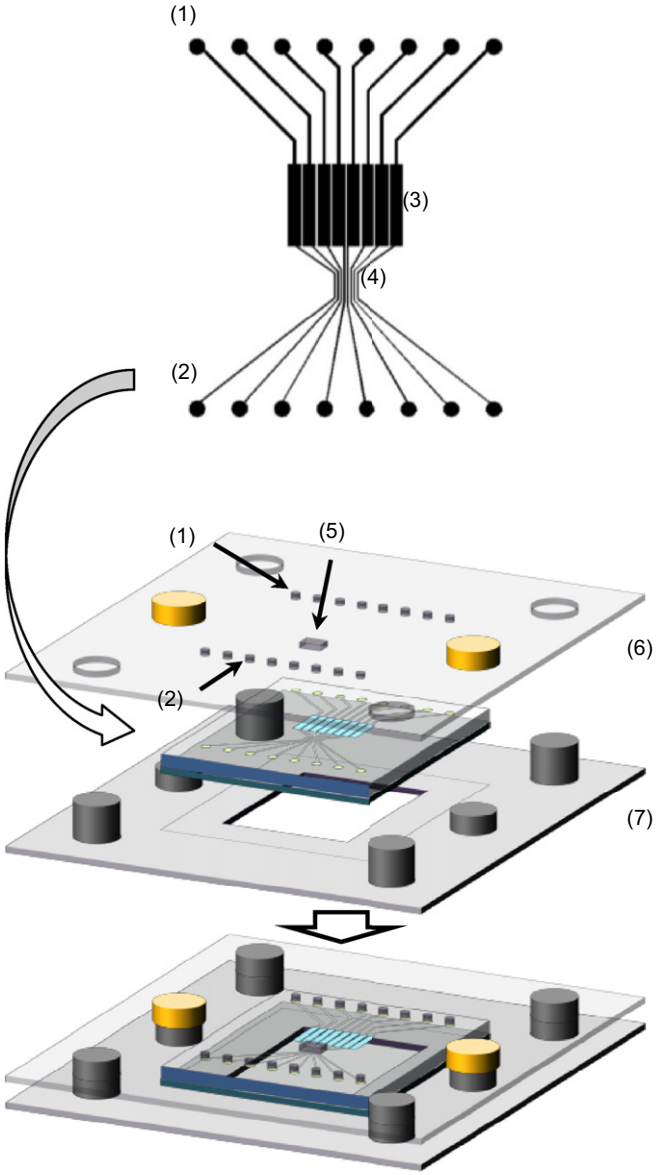

Fig. 2. Micro-fluidic PDMA platform containing PDMS microchip and jig system; 8 inputs (1), 8 outputs (2), 8 micro-reaction chambers (3), and 8 detection channels (4), the lid (6) containing the detection window (5), and bottom plate (7).

linearly proportional to the aptamer concentration in the range of 10^0–10^3 copies/5 μl, which was estimated as a possible range of aptamer concentration based on the size of a single micro-chamber in the micro-fluidic platform. As expected, nevertheless, the QNASBA efficiencies of the aptamer in the micro-chambers were a little bit lower than that in commercial tubes due to the microfluidics' characteristics (no convection flow). The linearity of QNASBA in this range was proven in both the commercial tubes and the PDMS micro-chambers. This result implies that the aptamer can play a role as a quantitative signal amplifier by QNASBA when the aptamer is utilized as a reporting probe. This aptamer employed for the quantification of monoclonal antibodies specific to markers (HER2, ER, PR, and ki-67) immobilized on the surface is shown in Fig. 3(b). It was amplified by QNASBA after washing the unbound aptamers on the monoclonal antibodies. Good linearity of T_T values on the marker antibodies was presented with R^2 values of 0.9996, 0.9999, 0.9972, and 0.9966 for

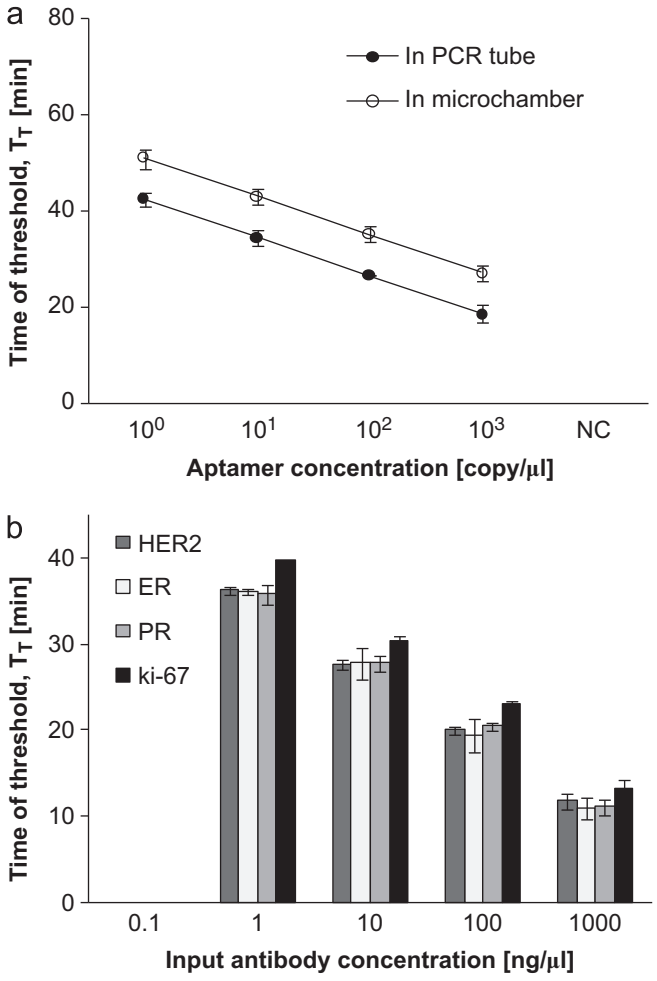

Fig. 3. Linearity of QNASBA for (a) aptamer concentration and (b) for 4 primary antibodies (HER2, ER, PR, and Ki67).

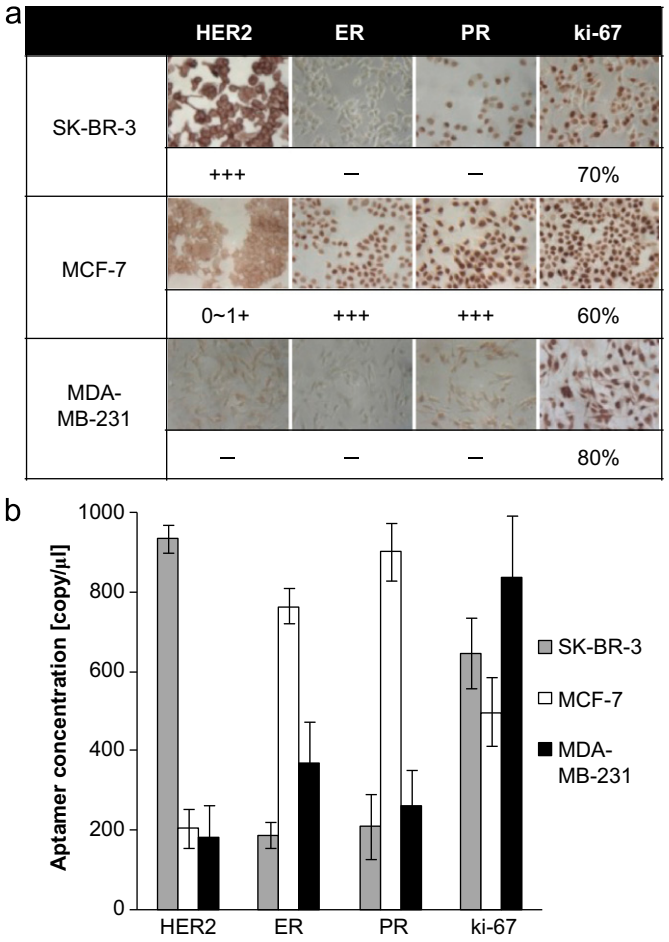

Fig. 4. Comparison of (a) conventional IHC and (b) AA-IHC on cell lines (SK-BR-3, MCF-7, MDA-MB-231).

monoclonal antibodies of HER2, ER, PR, and ki-67, respectively. This result showed that aptamer-assisted signal amplification in IHC can provide good quantitation of markers on real samples.

3.2. Quantitative and comparative analysis with IHC

To compare AA-QIHC with conventional IHC using HRP-labeled secondary antibody (as a control), the paraffin block side samples of SK-BR-3, MCF-7, and MDA-MB-231 were prepared, and their typical signals of conventional IHC were demonstrated (Figure S3). These conventional IHC results were summarized as shown in Fig. 4(a), which mostly corresponded with Subik's IHC results (Subik et al., 2010). However, the Ki-67 result in Fig. 4(a) was a little bit different from Subik's results in case of SK-BR-3. Our IHC result was moderately positive for ki-67, whereas it was negative (20%) in Subik's results. Another reference also reported that SK-BR-3 had only a few ki-67 markers (Kim et al., 2010). It was likely that cell characters depend on the cell's conditions, because Beinlich's result showed that SK-BR-3 had a somewhat higher signal than MCF-7's signal on ki-67 (Beinlich et al., 2000). Our conventional IHC results for the SK-BR-3 cell line on ki-67 markers showed a signal similar to that for the MCF-7 cell line. Therefore, we utilized these conventional IHC results on cell lines (Fig. 4(a)) as a control. The AA-QIHC was performed in the microfluidic platform. The microfluidic platform was placed on the paraffin block slides. It was pressured to prevent the leakage of buffer solutions from the micro-channels and chambers using a PMMA jig containing a detection window (Figure S4). The real-time result of the AA-QIHC on SK-BR-3 samples is shown in Figure S5. To calibrate the quantitative signals, the 3 different positive samples and 1 negative sample (1st–4th micro-chambers) were loaded and amplified coincidentally when aptamer bound on 4 primary antibodies in 4 different micro-reaction chambers were amplified (5th–8th micro-chambers). Figure S5 showed that SK-BR-3 cell lines were strongly positive for HER2 and slightly positive for ki-67, whereas negative signals were presented for ER and PR markers, respectively.

Fig. 4 (b) provides the quantitative results of the AA-QIHC. Comparing the AA-IHC result (Fig. 4(b)) with conventional IHC (Fig. 4(a)), the AA-IHC proposed in this study showed more precise and objective quantitative results using aptamer as a reporting probe. Particularly, AA-QIHC is valuable to quantify ki-67 markers because quantification of ki-67 markers is often so ambiguous with conventional IHC, compared to HER2, ER, and PR markers.

3.3. Real clinical sample analysis with aptamer combined IHC

To verify the clinical applications of the method proposed in this study, we applied this AA-QIHC to real clinical samples (Figure S6). Three samples were prepared with paraffin-blocked samples from 46–53 year-old patients who have 1.9–4 cm tumors (Stages I–III). Samples 1 and 2 were stored in 2010, whereas sample 3 was paraffin-blocked in 2004. Fig. 5(a) presents the

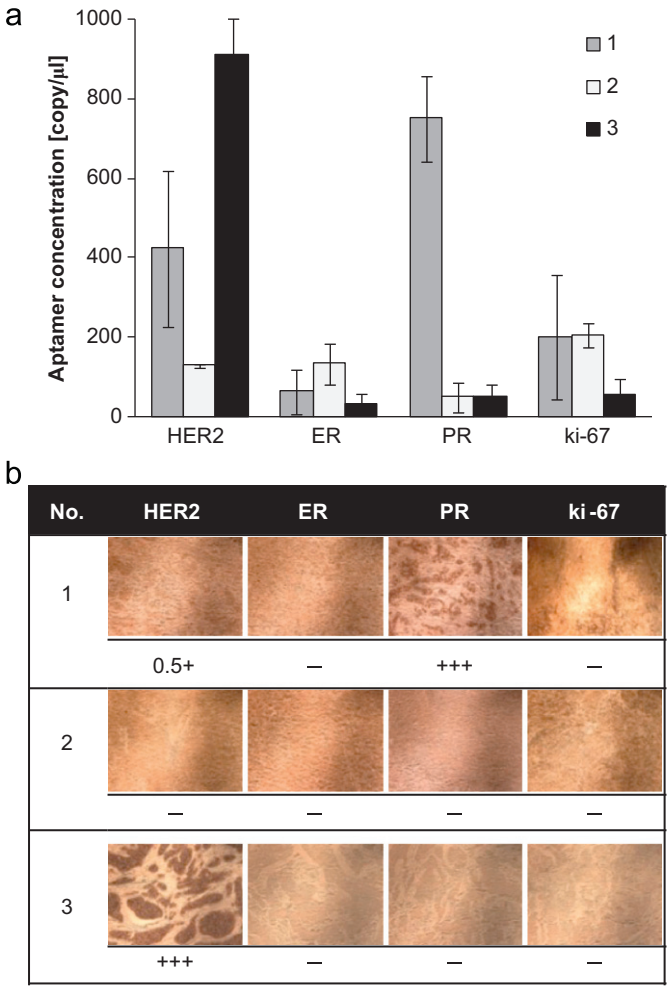

Fig. 5. Comparison of (a) AA-IHC results and (b) conventional IHC results on the real clinical samples.

results of AA-QIHC for the 3 samples, and Fig. 5(b) shows the conventional IHC results. Sample 1 had strong positive signals (almost 900 aptamer amplification) for PR markers and slightly positive signals (400 aptamer amplification) for HER2 and ki-67 markers. It was remarkable that the HER2 signal difference between sample 1 and sample 2 could be distinguishable by AA-QIHC, whereas they were not easily differentiated by the image results of conventional IHC. According to the conventional result, sample 1 might be diagnosed as luminal A, not luminal B, because HER2 marker appears negative by the conventional method. However, sample 1 was a subtype of luminal B according to the AA-QIHC.

These results of AA-QIHC corresponded to clinical results. Sample 1 was slightly positive for HER2 and strongly positive for PR (Luminal B); sample 2 was negative for all 4 markers (Triple Negative), and sample 3 was the only HER2 positive sample (HER2).

4. Conclusion

In this study, we have developed a simple and effective tool for the quantitation of marker protein in breast cancer tissue specimens using aptamer-assisted immunohistochemistry. The whole process for tumor cell characterization was reduced, and its quantitation was simple and efficiently performed in a microfluidic chip by AA-QIHC. The main issues associated with this technology were (1) the alternation of HRP-labeled secondary antibody to recognize primary antibodies specific to the target markers (HER2, ER, PR, ki-67) on the paraffin section of tumor tissue by aptamer, and (2) quantitation of aptamer bound to primary antibodies by its direct real time amplification in the presence on the paraffin section.

We developed a microfluidic plastic platform to perform the primary antibody reaction step, aptamer reaction step, and aptamer amplification step in a single chamber. By conducting these serial processes in a single chamber for each protein without the need to transfer samples to another chamber, no contaminants were present during the process. This simple method does not provide false negative results while still being highly sensitive. Thus, this tool holds promise for use in the development of commercialized IHC analysis systems to characterize breast cancer in clinical specimens.

Acknowledgments

This study was supported by the Industrial Core Technology Development Program of the Ministry of Knowledge Economy (no. 10037397), by the Original Technology Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (no. 2011-0027787), and by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (no. 2011-0022060).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.07.004.

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