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Communication

Aptamer based fluorescent probe for serum HER2-ECD detection: The clinical utility in breast cancer

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ABSTRACT

The transmembrane protein HER2 is overexpressed in approximately 30% of breast cancer patients. HER2-positive breast cancers tend to spread more aggressively, which result in increased mortality in women. Nowadays, the real-time monitoring of HER2 status is important in clinical diagnosis and treatment for HER2-positive patients. Although IHC and FISH assay are standard methods to evaluate the tissue HER2 status, both approaches which required high quality tissue samples and are not suitable for monitoring the status of HER2 in real time. Since extracellular domain (ECD) of the HER2 receptor can be shed into the circulation, the serum test of HER2 ECD has been developed as an additional approach to probe HER2 overexpression. The serum test will be able to monitor the dynamic changes of HER2 status. In this paper, we detected serum HER2 ECD using Cy5-labeled HB5 aptamer as a result of its specific binding ability to HER2 ECD. This aptamer-based fluorescent probe is easily synthesized and modified and as sensitive as anti-HER2 antibodies. We believe that Cy5-HB5 may have application potentials in serum HER2 test for clinical utility of breast cancer, such as recurrence and metastases.

Breast cancer, as the second most common malignant tumor and the fifth main reason of death from cancer, plays a great health problem all over the world. There is approximately 25% of all cancer patients suffer from breast cancer, which result in 15% cancer-related mortality in women [1]. Recently, Human epidermal growth factor receptor 2 (HER2) has been suggested as an important biomarker for breast cancer patients. HER2 is overexpressed in approximately 30% of breast cancer patients. Since HER2 is a protein that stimulates the growth of breast cancer cells, these HER2-positive breast cancers tend to grow and spread more aggressively [2,3]. Therefore, the early detection of HER2 protein is very important in the development and progression of certain aggressive types of breast cancer.

HER2, also known as Erb-B2, is a 185 kDa transmembrane protein belonging to the epidermal growth factor receptor family [4]. HER2 has been regarded as a tumor-specific target in molecular therapies for part of breast cancer patients. Nowadays, immunohistochemistry (IHC) assay and fluorescent *in situ* hybridization (FISH) assay are standard methods to evaluate the tissue HER2 status [5]. Only patients with a uniform intense membrane staining of more than 30% of invasive tumor cells on IHC, and a HER2-tochromosome 17 centromere (CEP17) ratio of greater than 2.2 on FISH are considered HER2-positive and are eligible for trastuzumab treatment [6]. However, there are numerous limitations on IHC and FISH methods. For example, both IHC and FISH have been shown to have false negative or positive results resulting from many reasons especially variations in assay protocol [7]. Growing evidence in recent years has revealed that not all the patients with HER2 positive status based on IHC or FISH test respond to trastuzumab [8]. Another limitation is the lack of "real-time" follow-up. During the treatment for HER2-positive patients, the clinician need to monitor HER2 status dynamically and change therapies appropriately [9,10]. Neither IHC nor FISH technique is a practical assay due to the requirement of high quality tissue samples, which may not always available. Thus, new technique needs to be developed to real-time monitor of HER2 status in the treatment for HER2-positive patients.

HER2 protein has three domains: an extracellular ligand-binding domain (ECD), a short transmembrane region, and an intracellular tyrosine kinase domain [11] as shown in Fig. 1. The

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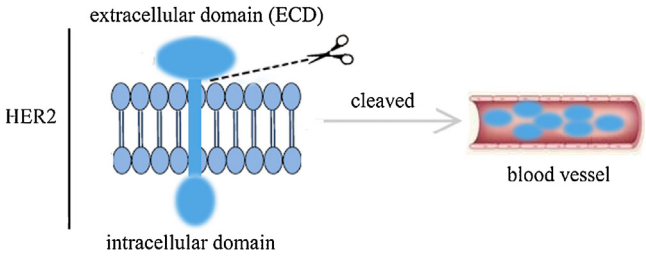

Fig. 1. The three domains of transmembrane protein HER2: an extracellular ligand-binding domain (ECD), a short transmembrane region, and an intracellular tyrosine kinase domain. The HER2 ECD domain was then released into the blood after being cleaved by proteolytic process.

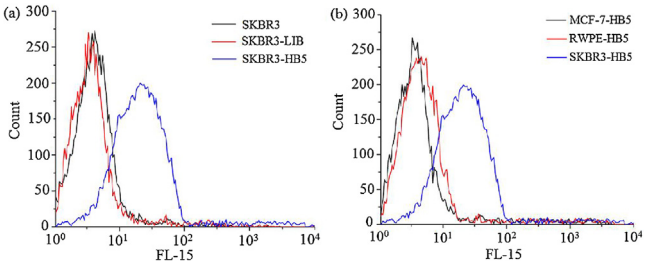

Fig. 2. The flow cytometric analysis of Cy5-HB5's selective binding property. (a) Flow cytometric spectrum of SKBR3 cells incubated with Cy5-HB5 and Cy5-LIB, respectively. (b) Flow cytometric spectrum of Cy5-HB5's bindings to HER2-positive breast cancer cells SKBR3, and HER2-negtive cancer cells MCF-7 and RWPE.

HER2 ECD domain will be released into the blood stream after being cleaved from the surface of cells by proteolytic process. Recent studies reported that HER2 ECD concentration in serum is related to tissue HER2 status and the detection of serum HER2 ECD can be used to optimize the identification of patients with/without HER2 overexpression in combination with IHC/FISH tests [12–17]. The HER2 ECD of serum is a noninvasive biomarker for detection of recurrence or metastasized and for predicting therapeutic response dynamically [4]. Meanwhile, the detection of HER2 ECD is a supplement of IHC or FISH after surgery. Enzyme-linked immunosorbent assay (ELISA) is a simple and useful method to measure serum HER2 ECD and have been wildly used [18]. However, the antibodies used in ELISA are heat-labile, prone to irreversible denaturation, expensive, and exhibit batch to batch variations [19]. The development of the substitution of antibodies is the new challenge for researchers.

In the past decades, several novel types of tumor-targeting ligands have been created besides antibodies [20]. One new class of ligands is aptamers, which are single-stranded oligonucleotides that may serve as targeting molecules in therapeutic and diagnostic applications [20]. The advantages of aptamers are high affinity, excellent specificity, and low immunogenicity. The synthesis and modification of aptamers are also easy [21]. Recently, a new HER2 aptamer (HB5) has been created as tumor-targeting ligand for delivering chemotherapeutic agent to HER2-positive breast cancer [22]. Furthermore, Wang and co-workers developed a novel IHC method using this DNA aptamer to evaluate the HER2 status *in situ* [23]. However, the HER2 aptamer (HB5) has not been studied in serum HER2 ECD testing. In this study, we tried to detect serum HER2 ECD using Cy5-labeled HB5 rather than specific anti-HER2 antibodies. The biological properties of HB5 based probe has been evaluated by the real-time detection of serum HER2 ECD specimens of breast cancer patients. Compared with conventional enzyme-linked immunosorbent experiment, HB5 based fluorescent probe has the huge potential for serum HER2 ECD detection in real-time and provides a new direction for the clinical diagnosis and treatment of breast cancer.

The sequences of Cy5-labeled HB5 (Cy5-HB5) aptamer is shown in Supporting information. We also designed and synthesized Cy5-labeled random DNA library (Cy5-LIB) as the control DNA sequence. In order to monitor the sensitivity and specificity of Cy5-HB5 aptamer, we incubated both Cy5-HB5 and Cy5-LIB to HER2-positive breast cancer cell SKBR3, and also incubated Cy5-HB5 to HER2-negtive cells, MCF-7 and RWPE. And then the cells were analyzed by flow cytometry. As shown in Fig. 2a, the fluorescence signal of the SKBR3 cells incubated by the Cy5-HB5 was significantly stronger than Cy5-LIB labeled SKBR3 cells. What is more, Cy5-HB5 showed relative stronger binding to HER2-positive breast cancer cell SKBR3, while the bindings to HER2-negtive cancer cells (MCF-7 and RWPE) were weak (Fig. 2b). The results showed that Cy5-HB5 was able to selectively bind to HER2-positive breast cancer cells and be possibly highly recognized by HER2 protein of breast cancer cells.

To further confirm the recognition ability of Cy5-HB5 to HER2 protein, the fluorescence images of SKBR3, MCF-7, and RWPE cells were observed after being incubated with Cy5-HB5 aptamer. As control, the fluorescence image of CY5-LIB labeled SKBR3 was observed, too. The signal of fluorescent probes is usually measured as a change in fluorescent intensity, fluorescent lifetime, or a shift of fluorescent wavelength [24,25], we used the fluorescent intensity to judge the ability of aptamer HB5 to identify the different expression of HER2 cell lines. As shown in Fig. 3, while HER2-negtive cells (MCF-7 and RWPE) and CY5-LIB labeled SKBR3 cells showed weak signals, only Cy5-HB5 labeled SKBR3 cells revealed red fluorescence signal. Furthermore, the fluorescence signal was showed on the surface of the SKBR3 cells. Since SKBR3 cells overexpressed HER2 receptor with extracellular domain ECD exposing on the surface of cells, the result demonstrated that Cy5-HB5 aptamers were able to bind with HER2 ECD specially. It was conform with the study of Yang and co-worker as described in Ref. [22].

The results of flow cytometry and confocal fluorescence microscopy analysis have confirmed that aptamer Cy5-HB5 can identify the different expression of HER2 cell lines with specific and sensitive recognition capability to HER2 ECD. Furthermore, we tried to detect serum HER2 ECD using Cy5-HB5 aptamer. This study was undertaken with the aim to explore the clinical utility of serum HER2-ECD estimation by Cy5-HB5 aptamer in breast cancer patients and evaluate whether it could be used in place of antibodies for diagnostic purpose.

The method we detecting serum HER2-ECD with Cy5-HB5 aptamer was similar to ELISA protocol as described in Supporting information. First, we intended to make a standard curve with pure HER2-ECD samples using Cy5-HB5 aptamer and anti-HER2 antibody, respectively. Different concentrations of HER2-ECD protein were used to be tried in this experiment. Finally, as shown in Fig. 4, the standard curve was gained with concentrations range of the HER2-ECD protein from 1.5 μg/L to 24 μg/L. The great linear correlation of the standard curve ($R^2 = 0.9904$ for antibody and $R^2 = 0.9939$ for Cy5-HB5 aptamer) indicated that we can detect serum HER2-ECD using either antibody or Cy5-HB5 aptamer quantitatively.

We evaluated biological properties of aptamer and antibody by detecting the serum specimens of 30 breast cancer patients. We detected serum HER2 ECD with either HER2 antibody or aptamer HB5 to evaluate sensitivity and specificity of the two methods. The HER2 ECD concentration of each sample is calculated according to the standard curve formula (Fig. 4). We used a cut-off value of 24.5 ± 6.2 μg/L to consistent with ELISA. As shown in Table 1, there are conformance results of 7 cases of HER2 (+) and 20 cases of HER2 (−) in totally 30 serum specimens of breast cancer patients that detected by Cy5-HB5 aptamer and anti-HER2 antibody,

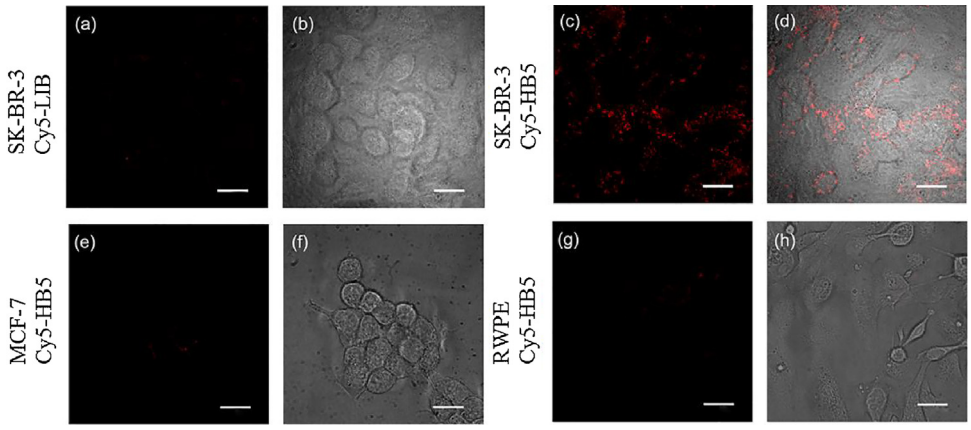

Fig. 3. The detection of HB5's specific binding property with Laser confocal microscopy. Fluorescence images of HER2-positive breast cancer cell, SKBR3, treated with Cy5-LIB or Cy5-HB5 were shown in (a, b) and (c, d), respectively. Cy5-HB5 was then incubated with HER2-negtive cells, MCF-7 and RWPE, as shown in fluorescence images of (e, f) and (g, h). Among them, (a), (c), (e), and (g) are fluorescence images, excitation wavelength is 640 nm. And (b), (d), (f), and (h) are merged images of both fluorescence and bright-field images. Scale bar = 20 μm.

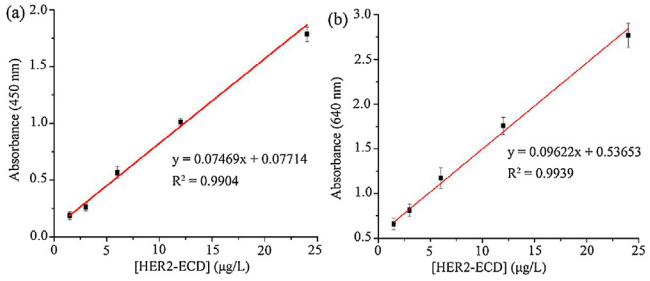

Fig. 4. Standard curves of pure HER2-ECD detection using HER2 antibody (a) and Cy5-HB5 aptamer (b), respectively.

Table 1
The comparison of serum HER2 ECD detection between Cy5-HB5 and antibody methods.

Serum HER2 ECD (Cy5-HB5)	Serum HER2 ECD (antibody)		
	Positive	Negative	Total
Positive	7	2	9
Negative	1	20	21
Total	8	22	30

respectively. The testing of serum HER2 ECD by aptamer HB5 had a high statistical correlation with that detected by anti-HER2 antibody calculated by GraphPad Prism 5 (Fisher's exact test, $P < 0.05$, Table 1). It demonstrated that the sensitivity and specificity of Cy5-HB5 aptamer is closely related to anti-HER2 antibody. So HB5 based fluorescent probe has the huge potential for serum HER2 ECD detection in real-time.

Furthermore, we evaluate the relationship between serum HER2 ECD levels and clinicopathological characteristics. The HER2 expression of serum detected by anti-HER2 antibody was compared with the HER2 expression of tissue. We observed that serum HER2 ECD levels detected by anti-HER2 antibody had a correlation with tissue HER2 status confirmed by IHC/FISH ($P < 0.05$, Table 2) and the specificity and sensitivity of serum HER2 tested by anti-HER2 antibody were 95% and 70% defined by IHC/FISH. And the HER2 ECD of serum detected by HB5 was compared with tissue HER2 status confirmed by IHC/FISH. The results showed that serum HER2 ECD levels detected by aptamer HB5 also had a correlation with tissue HER2 status confirmed by IHC/FISH ($P < 0.05$, Table 2) and the specificity and sensitivity of serum HER2 tested by HB5 were 100% and 90% defined by IHC/FISH. Aptamer HB5 has a higher specificity and sensitivity than anti-HER2 antibody. Meanwhile, we analyzed the relationship between serum HER2 ECD levels and other clinicopathological characteristics. We found that high serum HER2 ECD levels detected by anti-HER2 antibody and HB5 were significantly correlated with tumor size, lymph nodes status ($P < 0.05$, Table 2). in addition, no correlation was found between high serum HER2 ECD levels detected by anti-HER2 antibody and HB5 and age, ER status, PR status ($P > 0.05$, Table 2).

In our study, the concentration of serum HER2 ECD were obviously lower in patients with negative tissue HER2 status detected by IHC than that in patients with positive tissue HER2 status confirmed by IHC/FISH. Serum HER2 detected by anti-HER2 antibody had a high statistical correlation with tissue HER2 status ($P < 0.05$) and serum HER2 detected by HB5 came to the same conclusion ($P < 0.05$). Serum HER-2 concentration is related to tissue HER2 status and the serum HER2 can be used to optimize the identification of patients with HER2 overexpression in combination with IHC/FISH tests. Our results are consistent with other studies [12–17]. And the testing of serum HER2 by aptamer HB5 had a high statistical correlation with that detected by anti-HER2 antibody ($P < 0.05$). However, the aptamer HB5 had a high specificity (100% *vs.* 95%) and sensitivity (90% *vs.* 70%) than anti-HER2 antibody in the detection of serum HER2. In addition, the results of flow cytometric and laser confocal microscopy indicated that aptamer HB5 had the high selective and specific ability to recognize the HER2 positive cells and won't produce cross-reactivity with other cell lines. We proved that aptamer HB5 has the similar ability to detect serum HER2 ECD level than anti-HER2 antibody. So, Cy5-HB5 is a potential substitution of antibody in serum HER2 ECD detection.

In conclusion, we proposed a novel serum HER2-ECD test method using aptamer Cy5-HB5 as fluorescence probe. Cy5-HB5 can not only identify the HER2 overexpression in breast cancer cell lines, but also recognize HER2-ECD in serum quantitatively. Through the detection of serum specimens of 30 HER2-positive/negative breast cancer patients, we found the sensitivity and specificity of Cy5-HB5 probe to serum HER2-ECD can compete with HER2-antibody used in HER2 human ELISA kit. Since aptamer is easily synthesized and modified, HB5 aptamer can be used as a powerful substitution of HER2-antibody in the real-time serum HER2-ECD test for the better supporting the clinical diagnosis and treatment of breast cancer.

Table 2
Relationship between serum HER2 ECD levels, tissue HER2 status and clinicopathological characteristics.

Characteristics	Serum HER2 status					P (Cy5-HB5)
	Positive	Negative	P (antibody)	Positive	Negative	
Tissue HER2 status						
Positive	7	3	0.0004	9	1	<0.0001
Negative	1	19		0	20	
Age (years)						
≥40	6	10	0.2255	7	9	0.1184
<40	2	12		2	12	
Tumor size (cm)						
>2	7	9	0.0395	8	8	0.0169
≤2	1	13		1	13	
Lymph nodes status						
Positive	6	3	0.0031	7	4	0.0042
Negative	2	19		2	17	
ER status						
Positive	5	13	1.0000	6	12	0.7036
Negative	3	9		3	9	
PR status						
Positive	5	13	1.0000	6	12	0.7036
Negative	3	9		3	9	

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References

[1] L.A. Torre, F. Bray, R.L. Siegel, J. Ferlay, CA: Cancer J. Clin. 65 (2015) 87–108.
[2] T. Cooke, J. Reeves, A. Lanigan, P. Stanton, Ann. Oncol. 12 (Suppl. 1) (2001) S23–S28.
[3] T. Holbro, G. Civenni, N.E. Hynes, Exp. Cell Res. 284 (2003) 99–110.
[4] L. Lam, N. McAndrew, M. Yee, et al., Biochim. Biophys. Acta 1826 (2012) 199–208.
[5] A.C. Wolff, M.E. Hammond, D.G. Hicks, et al., Arch. Pathol. Lab. Med. 138 (2014) 241–256.
[6] A.C. Wolff, M.E. Hammond, J.N. Schwartz, et al., Arch. Pathol. Lab. Med. 131 (2007) 18–43.
[7] E.A. Perez, V.J. Suman, N.E. Davidson, et al., J. Clin. Oncol. 24 (2006) 3032–3038.
[8] L. Gianni, T. Pienkowski, Y.H. Im, et al., Lancet Oncol. 13 (2012) 25–32.
[9] U. Wilking, E. Karlsson, L. Skoog, et al., Breast Cancer Res. Treat. 125 (2011) 553–561.
[10] N.H. Turner, A. Di Leo, Cancer Treat. Rev. 39 (2013) 947–957.
[11] C. Tse, A.S. Gauchez, W. Jacot, P.J. Lamy, Cancer Treat. Rev. 38 (2012) 133–142.
[12] W.J. Kostler, B. Schwab, C.F. Singer, et al., Clin. Cancer Res. 10 (2004) 1618–1624.
[13] R. Molina, J. Jo, X. Filella, et al., Breast Cancer Res. Treat. 51 (1998) 109–119.
[14] K. Sugano, M. Ushiama, T. Fukutomi, et al., Int. J. Cancer 89 (2000) 329–336.
[15] M. Krainer, T. Brodowicz, R. Zeillinger, et al., Oncology 54 (1997) 475–481.
[16] C. Mazouni, A. Hall, K. Broglio, et al., Cancer 109 (2007) 496–501.
[17] Y. Kong, S. Dai, X. Xie, et al., J. Cancer Res. Clin. Oncol. 138 (2012) 275–284.
[18] T. Fehm, P. Maimonis, S. Weitz, et al., Breast Cancer Res. Treat. 43 (1997) 87–95.
[19] A. Sett, B.B. Borthakur, U. Bora, Clin. Transl. Oncol. 19 (2017) 976–988.
[20] A.S. Barbas, J. Mi, B.M. Clary, R.R. White, Future Oncol. 6 (2010) 1117–1126.
[21] M. Famulok, J.S. Hartig, G. Mayer, Chem. Rev. 107 (2007) 3715–3743.
[22] Z. Liu, J.H. Duan, Y.M. Song, et al., J. Transl. Med. 10 (2012) 148–158.
[23] M. Chu, J.R. Kang, W. Wang, et al., Cell Mol. Immunol. 14 (2017) 398–400.
[24] S. Leng, Q.L. Qiao, L. Miao, et al., Chin. Chem. Commun. 28 (2017) 1911–1915.
[25] Y. Yang, H. Wang, Y. Wei, et al., Chin. Chem. Lett. 28 (2017) 2023–2026.