Original Article
Detection of HER2 positive circulating tumor cells using anti-HER2-renilla luciferase fusion protein

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Abstract: Recent progress in cancer research has demonstrated that circulating tumor cells (CTCs) can be used as easily detectable biomarkers with high prognostic value allowing the early detection of metastasis in breast cancer (BC). The aim of this study was to establish a new HER2+ CTC detection assay that might provide a valuable reference for improving diagnosis and guiding treatment decisions. This assay involves the use of a bioluminescent protein sequence from the sea pansy, *Renilla reniformis*, conjugated to a single chain variable fragment (scFv) of the HER2 antibody. This chimeric anti-HER2-Renilla luciferase fusion protein (anti-HER2-RLuc) targets HER2 cell surface antigens on CTCs and can be activated to emit light. Anti-HER2-RLuc was able to identify low numbers of HER2+ SK-BR-3 cells (fewer than 10) mixed with large numbers of MDA-MB 231 cells, and this method was also successfully used to detect HER2+ CTCs in blood samples from BC patients (n=59). A lower anti-HER2-RLuc signal, which suggests a lower CTC level, could be detected in five BC patient blood samples after surgery compared to preoperative patients. Similar results were also obtained with cell medium-derived exosomes. However, *in vivo* optical imaging of tumor-bearing athymic mice failed to demonstrate specific targeting of anti-HER2-RLuc to HER2+ xenografts. We also used protein A/G-coated plates to successfully screen HER2+ SK-BR-3 cells mixed with HER2- MDA-MB 231 cells, and immunofluorescence (IF) staining of HER2+ cells further confirmed the results. The scFv-luciferase-based detection assay has the potential to be a practical assay that can be used to facilitate targeted treatment in HER2+ breast cancer.

Keywords: Circulating tumor cells, breast cancer, anti-HER2-RLuc, bioluminescence

Introduction

Breast cancer (BC) is the most common malignant cancer found in women [1]. The overexpression of the human epidermal growth factor receptor 2 (HER2) is detected in 15-20% of breast cancers patients [2-4] and, due to a point mutation and augmented expression, is associated with resistance to many types of treatment and a poor prognosis [4, 5]. The circulatory system provides a route for the transmission of breast cancer cells to secondary proliferating metastatic lesions, and high CTC counts are associated with a poor prognosis

and have predictive value in early disease [6-10]. Over the past few years, researchers have found that CTCs in peripheral blood (PB) express HER2 levels corresponding to those in samples from primary and metastatic sites [11, 12]. In addition, other studies have demonstrated that due to heterogeneity and several potentially relevant mutations related to BC, the expression of HER2 on CTCs is often identified in patients with HER2- primary tumors after multiple courses of therapy [13-15]. Therefore, better comprehensive characterization of CTCs in real time might provide more precise treatments for individual patients.

In this study, we aimed to use a safe, cost-effective, specific and highly sensitive novel detection technique based on single-chain variable fragment (scFv)-luciferase protein to evaluate the level of HER2 in CTCs. Here, we used the bioluminescent protein sequence from *Renilla reniformis* conjugated to a scFv of the HER2 antibody. This scFv-luciferase fusion protein can target CTC-specific/exosome surface antigens and transmit an amplified signal for simple detection that can be performed on a large scale.

Furthermore, we used another technology involving protein A/G-coated plates to screen HER2^{+} cells from a large population of background cells. Gene fusion of the Fc-binding domains of protein A and protein G produced a structural and functional chimeric protein with a broader binding capacity than either protein A or protein G alone. Protein A/G can bind to many human Ig subclasses and scFv fragments. Based on this principle, we hypothesized that HER2^{+} CTCs will first bind to anti-HER2-RLuc, and when added to protein A/G-coated plates, anti-HER2-RLuc will bind to protein A/G. To test the feasibility of this method, we used cell-based experiments and clinical blood samples.

Exosomes are endosome-derived nanovesicles have a specific physical structure and are small in size. Exosomes secreted by cancer cells contain specific antigens that are potentially useful for immunodetection [16, 17]. In this study, we also detected exosomes released by HER2-overexpressing carcinoma cells and explored the possibility that anti-HER2-RLuc might detect HER2^{+} exosomes, which are nanovesicles.

To the best of our knowledge, this investigation constitutes the first systematic study using this new detection method that can effectively detect HER2^{+} CTCs and exosomes. These data provide the basis for further investigation of the detection capacity of scFv-luciferase fusion proteins.

Materials and methods

Cell cultures

All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and were used for the CTC detection experiments. The SK-BR-3 adenocarcinoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco), and the MDA-MB-231 and K562 cell lines were cultured in RPMI 1640 (Gibco). Both media were supplemented with 10% fetal calf serum (FCS) (Sigma) and 1% penicillin/streptomycin (Gibco). The cells were maintained under standard conditions at 37°C in a humidified atmosphere of 5% CO_2.

Construction of the anti-HER2-Renilla luciferase plasmid, transfection, and measurements of RLuc activity: The anti-human HER2 single-stranded antibody gene was synthesized by Life Technology. The Renilla luciferase gene was cut with restriction enzymes (NheI/XbaI) from the pRL-TK vector (Promega Corporation, Madison, WI, USA) and then subcloned into a pcDNA3 expression vector. The anti-HER2 gene was then subcloned into the pRL-TK vector at the N terminus of the RLuc gene (to maintain the reading frame) using the HindIII/Nhe1 sites. The anti-HER2-Renilla luciferase plasmid was transfected into 293FT cells in 10-cm cell culture dishes with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommended protocol. After 48 h, the medium was discarded, and the cells were gently washed twice with cold PBS. The cells were lysed with 10 mL of passive lysis buffer (PLB, Promega) for 30 min at room temperature, as recommended by the manufacturer. Luminometry was performed in an LB942 luminometer (Berthold Detection Systems, Germany). The light output was measured for 10 seconds and expressed as relative light units (RLUs).

Cell experiments

Binding assay to MDA-MB-231 and SK-BR-3 cells: To verify the feasibility and binding capacity of the anti-HER2-Renilla luciferase fusion protein, we generated a gradient dilution (0, 10, 100, 1000 and 10,000 cells) of two human breast carcinoma cell lines (HER2^{-} MDA-MB-231 and HER2^{+} SK-BR-3), as detected by western blotting. The cells were incubated with anti-HER2-RLuc (1×10^8 RLU) for 3 h and were then washed three times by centrifugation. Finally, the cells were resuspended in 50 µL of PBS and measured using an LB942 luminometer.

Table 1. Characteristics and pathological features of the patients

Characteristics	n (%)
Age	
Mean	54
Range	28-86
Sex	Female
Histological type	
Intraductal carcinoma	1 (1.7%)
High grade intraductal carcinoma	3 (5.1%)
Ductal carcinoma	52 (88.1%)
Lobules carcinoma	2 (3.4%)
Clinging carcinoma	1 (1.7%)
IHC (HER2 score)	
3+	12 (20.4%)
2+	16 (27.1%)
1+	15 (25.4%)
0	16 (27.1%)
IHC (Hormone receptor)	
ER+ PgR+	23 (39%)
ER+ PgR-	11 (18.6%)
ER- PgR+	1 (1.7%)
ER- PgR-	24 (40.7%)
Metastatic lymph nodes	
Negative	31 (52.5%)
Positive	28 (47.5%)
Distant metastasis	0

ER, estrogen receptor; PgR, progesterone receptor.

Specificity of the anti-HER2-Renilla luciferase fusion protein: To identify the binding specificity, 10,000 SK-BR-3 cells were incubated with a commercial anti-HER2 antibody at different dilution concentrations (0, 1:100,000, 1:10,000, 1:1000, and 1:100) for 2-3 h and then washed three times with PBS to remove unbound antibody. The cells were then resuspended in 1 mL of PBS, and anti-HER2-RLuc (10^8 RLU) was added to the cell suspension. After incubation for another 3 h, the cells were washed three times by repeated resuspension in 1 ml of PBS. Luminometry was performed using an LB942 luminometer.

Sensitivity of the anti-HER2-Renilla luciferase fusion protein: Mixed cell experiments were used to detect the sensitivity of anti-HER2-RLuc. A gradient dilution (0, 30, 60, 125, 250, 500 and 1000) of individual cell lines was used for the first two groups, whereas in the third group, the same gradient dilution of SK-BR-3 cells was mixed with a fixed number (1×10^4) of MDA-MB-231 cells. Anti-HER2-RLuc (1×10^8 RLU) was added, and luminometry was performed as previously described.

Optical imaging of tumor-bearing mice

All animal handling was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Laboratory Animals Care and Use Committee of the Second Hospital of Shandong University. After 10 days of acclimatization, tumors (P1) were induced using the SK-BR-3 and MDA-MB-231 cell lines ($1-2 \times 10^6$ cells) in 6- to 8-week-old female athymic mice purchased from the Model Animal Research Center of Nanjing University (n=8). At time zero, the mice were injected via the tail vein with 100 μL of anti-HER2-RLuc (1×10^8 RLU). After 2, 4, 6, and 8 h, the mice were anesthetized with isoflurane, which was injected via the tail vein with 100 μL of coelenterazine (3 mg per 20 g of mouse weight), and immediately imaged for bioluminescence (Perkin Elmer). Tumor masses were allowed to develop for 60 days and reached 140-200 mg. The mice were then euthanized by CO_2 administration, and the tumors were removed, placed in ice-cold PBS and then cut into small pieces. We subsequently anesthetized another eight athymic nude female mice, and a small incision was made on the right side of the axilla. After transplantation of the tumors, the skin was closed with a suture. The animals were monitored every three days for primary tumor growth (P2 tumor volume). For tumor volume measurement, a vernier caliper was used to measure the longest and shortest diameters of the tumor. At the end of the experiment, the whole blood was collected directly into 2 ml EDTA vacutainer tubes (Becton Dickinson) by cardiac puncture using a 1 mL needle.

Patients and clinical samples

59 breast cancer patients who had not received any treatment and who presented with stage I-III invasive breast cancer with locally advanced or lymphatic metastatic disease were eligible for this research. A separate control group was comprised of 14 women with benign breast diseases. The clinical and pathological features of the primary tumors are provided in **Table 1**. All the patients were given information regarding the experimental design of the study and provided written informed consent prior to collec-
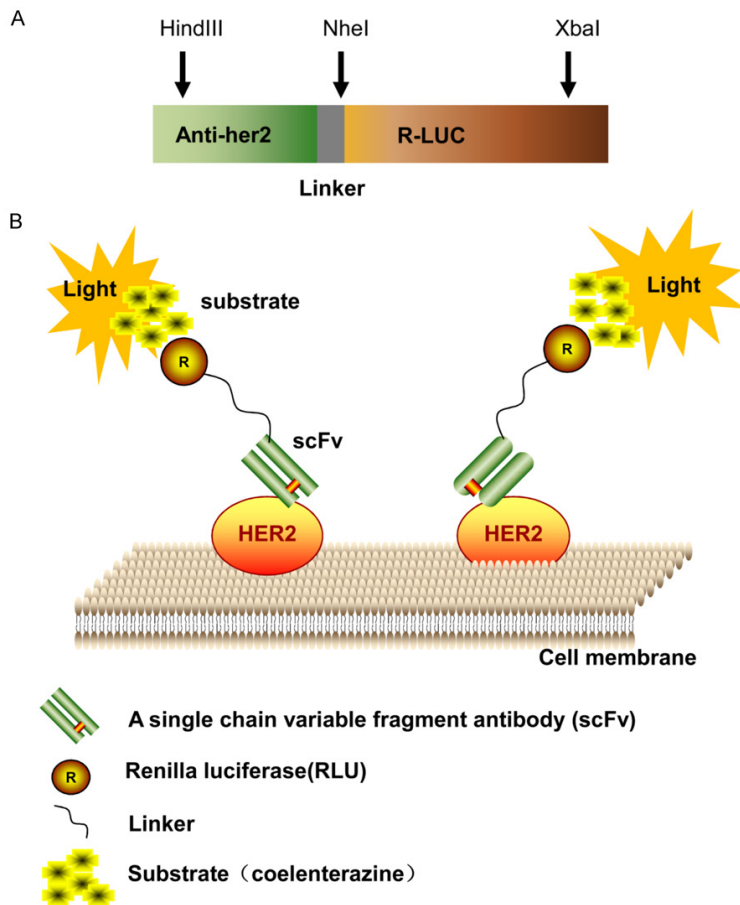
Figure 1. Schematic diagram of the anti-HER2-RLuc fusion protein detection system. A. Schematic diagram of genes encoding the anti-Her2-RLuc fusion protein. scFv is composed of the light and heavy chain variable regions joined by an amino acid linker, and this sc-FV was joined to the Renilla luciferase gene by the indicated ctggtggtgggagcc amino acid linker. B. Process of the detection of this new technique-scFv-luciferase protein. HER2-positive breast cancer cells can be specifically identified with anti-HER2-RLuc fusion proteins, which can simultaneously react with coelenterazine and emit light.

patients, we also collected clinical carcinoma tissue specimens following surgery. These tissues were flash-frozen in liquid nitrogen, and stored long-term at -80°C.

Isolation of PBMCs with Ficoll density gradient

Blood samples (from both patients and mice) were subjected to enrichment and CTC detection. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque according to the manufacturer's instructions.

Screening of CTCs

In addition to the CTC detection protocol mentioned above, we conducted further CTC screening. A fixed number of MDA-MB-231 cells ($1×10^5$) were added to varying numbers of SK-BR-3 cells (0, 80, 400, 2000, 4000, 20000 and 40000 cells per 1 mL of serum-free DMEM) and then incubated with anti-HER2-RLuc ($1×10^8$ RLU). After 3 h of incubation on a shaker at room temperature, the cells were washed three times with PBS. The supernatant was removed, and the cells were resuspended in 200 μL of serum-free DMEM and then transferred to a protein A/G-coated plate (Thermo Fisher). The plates were then incubated for another 3 h and gently washed four times with PBS, and after the cells were resuspended in 50 μL of PBS in each well, the light signal was detected using an LB942 luminometer.

Exosome detection

All cells were seeded at a density of $1×10^5$ cells/mL in fresh complete RPMI medium containing FBS. After 24 h, exosome-containing culture medium was collected. The medium was centrifuged at 2,000 g for 30 min to
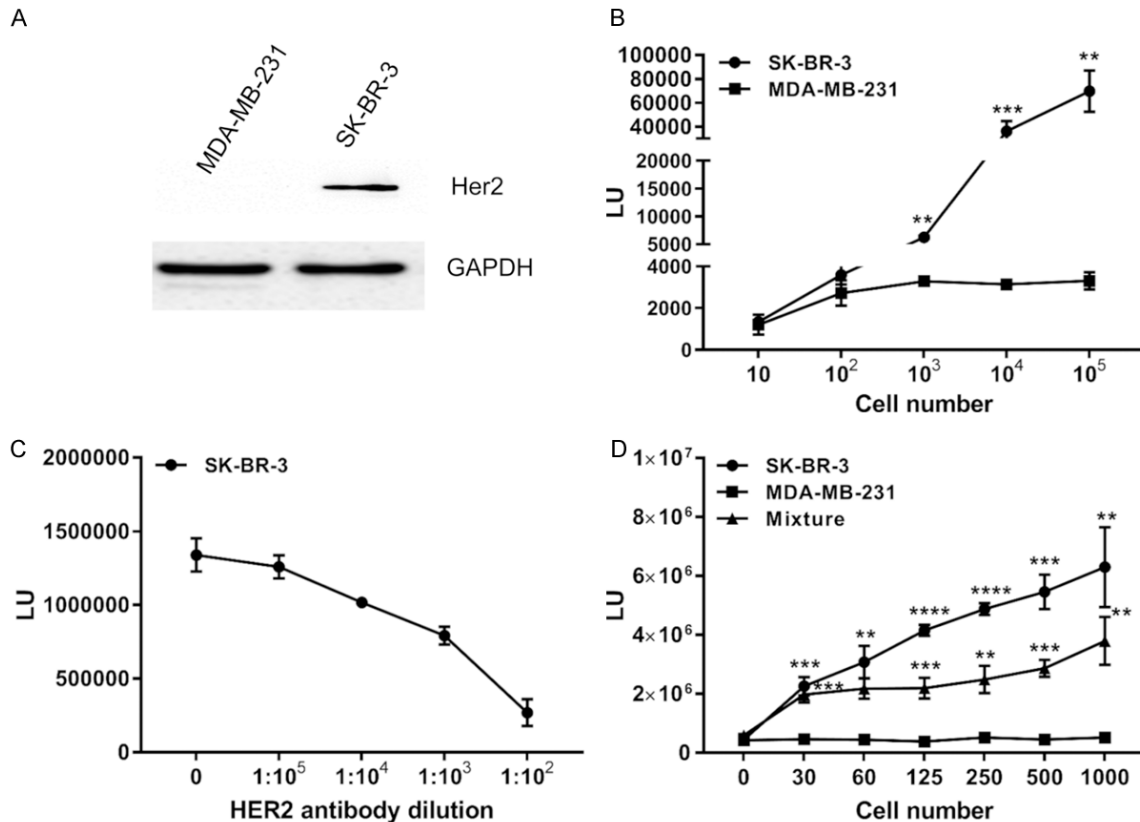
tion of blood for CTC analyses. This study was approved by the Second Hospital of Shandong University Ethics Committee and strictly followed the principles listed in the Declaration of Helsinki. Patients who had received previous treatments or who had not been pathologically examined after diagnosis were excluded from this study.

5 mL samples of peripheral blood were collected into EDTA vacutainer tubes (Becton Dickinson). The samples were maintained at room temperature and processed within 4 h from collection. In addition to the collection of the fresh blood samples of breast cancer

Figure 2. Specificity and sensitivity of anti-HER2-RLuc for detection of HER2⁺ cells. A. Western blotting was used to detect the HER2 expression of SK-BR-3 and MDA-MB-231cells. B. In order to text the feasibility of this method, we incubated anti-HER2-RLuc with these two cell lines separately, and detected the light signal using an LB942 luminometer. C. We then used a commercial HER2 antibody as a competitive reagent to the fusion protein to demonstrate the specificity of anti-HER2-RLuc. D. We mixed varing numbers of SK-BR-3 cells and MDA-MB-231 cells to determine the sensitivity of anti-HER2-RLuc. The data presented are representative of three independent experiments ($P < 0.05$, n=3).

remove any whole cells or debris. Culture medium (1 mL) from the indicated cell types was incubated with 5 µL of HER2-Luc protein (1×10^8 RLU/µL) for 1 h at 4°C. The Invitrogen Total Exosome Isolation reagent was then used to isolate exosomes (from cell culture media) according to the manufacturer's recommended protocol with minor modifications. The exosome reagent (0.5 mL) was added to each sample and then centrifuged at 10,000 g for 10 min. The samples were washed 3 times by removing the supernatant, resuspending the exosome-containing pellet in 200 µL of PBS, adding 100 µL of exosome reagent for a 30-min incubation, and centrifuging at 10,000 g for 10 min. The washed pellet was then resuspended in 50 µL of PBS and mixed with luciferase substrate, and the bioluminescence was measured in an LB942 luminometer (Berthold Detection Systems, Germany).

To explore the existence of a positive correlation between the light signal and the volume of cell culture medium, the indicated volume of culture medium was used for detection.

Western blotting

Monoclonal antibodies against HER2 and GAPDH (Abcam) were used for western blotting. Protein expression was determined through enhanced chemiluminescence (ECL) (Millipore).

Immunofluorescence (IF) staining of HER2⁺ CTCs

Cells were first incubated with goat serum blocking solution (ZSGB-BIO, ZLI-9056) for 1 h and then incubated with an anti-HER2 antibody (Abcam, ab13418) at a 1:300 dilution overnight at 4°C. After washing, the cells were incu-
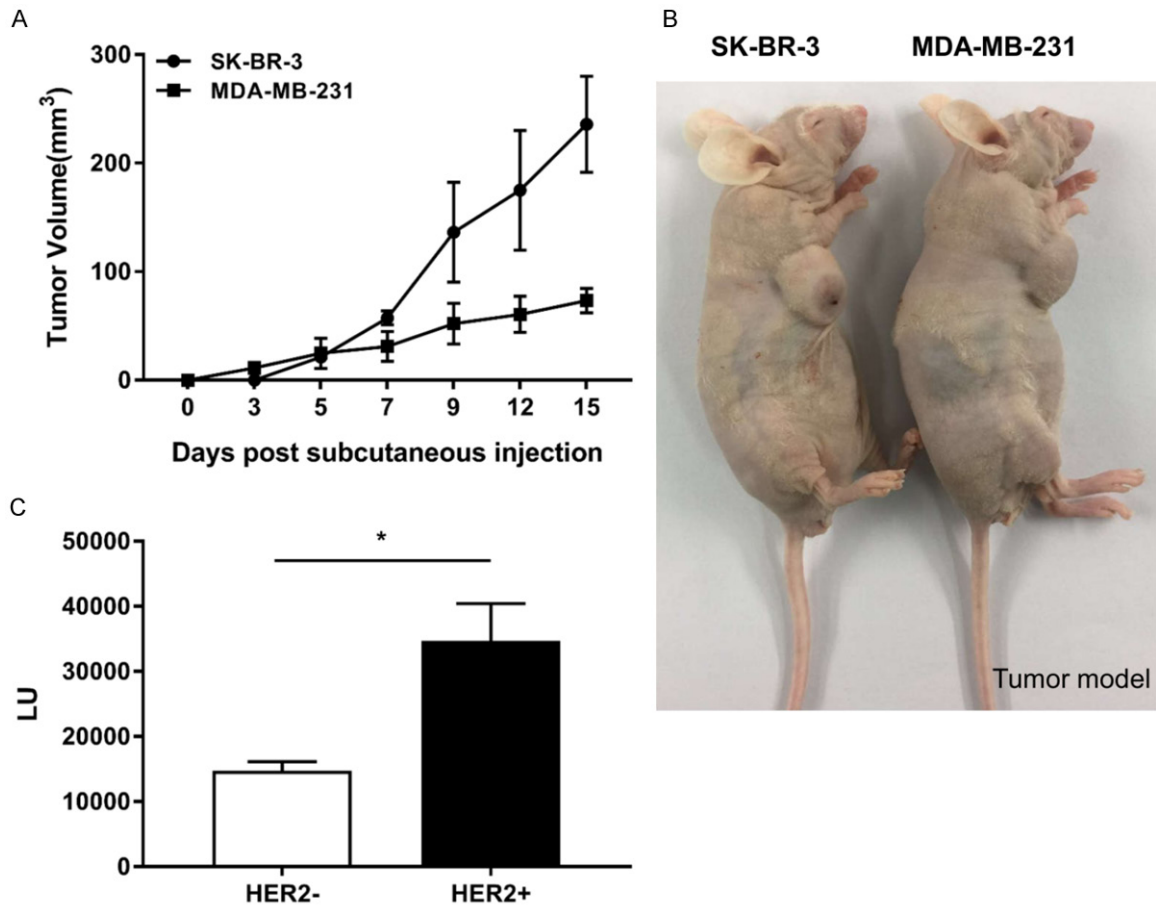
Figure 3. Tumor grafts in mouse model experiments. A. The volume was measured every other day for two weeks. The diagram shows the tumor growth curve of the eight mice at the indicated times after i.v. injection of the two tumor cell lines. Points, means; bars, SDs. (n=4 per group). B. Images of the tumors on the right shoulders of the athymic nude mice following i.v. injection of SK-BR-3 and MDA-MB-231 cell lines. C. Blood samples were harvested from mice four weeks after tumor grafts. The anti-Her2-RLuc fusion protein was used to detect blood CTCs from HER2$^+$ and HER2$^-$ inoculated mice (n=8).

bated with an Alexa Fluor 488-conjugated secondary antibody (1 h, 22°C). The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI).

Statistical analysis

The statistical significance of the differences in the light signal among multiple groups (human blood samples) was analyzed by one-way ANOVA method using SPSS 7.5 statistical software. Data are presented as the means ± SDs, and P<0.05 was considered significant. Statistical analyses comparing the results between two groups (cell experiment, mouse experiment, and protein A/G screen experiment) were performed by one-way ANOVA followed by Student's t-test using Prism 5 (GraphPad So-

ftware, Inc.). Moreover, *, **, and *** indicate statistically significantly (P<0.05, P<0.01, and P<0.001, respectively) compared with the control. Each experiment was conducted in triplicate.

Results

Expression and construction of scFv-RLuc fusion proteins

A scFv antibody is a fusion protein composed of variable regions of the light and heavy chains of immunoglobulin and is used for antigen recognition. The sequences of scFvs are directed against specific CTC surface antigens such as HER2 and can be cloned into CMV expression vectors upstream of the Renilla luciferase

Table 2. HER2 status of BC and BF patients assessed by an LB942 luminometer

IHC	HER2 score				
	3+	2+	1+	0	BF
Mean	997964.6	686390.8	321045.8	480112.4	170800.6
SD	612057.6	573131.1	370443.6	395019.7	143556.6
N	12	16	15	16	14

The relationship of the luminescence results among the five groups were analyzed by Analysis of Variance (ANOVA), $P < 0.05$ were considered statistically significant. HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

sequence. The transfection and expansion of cells can rapidly yield high quantities of scFv-luciferase fusion proteins (**Figure 1A**). A short linker peptide separates the scFv from the luciferase reporter, rendering a functional protein that can bind a specific antigen and emit a bioluminescent signal when activated. The ultimate purpose is to develop a signal assay used to identify the presence of a specific type of breast cancer in the early stage of the disease, as shown in the schematic diagram in **Figure 1B**.

In vitro validity, specificity, and sensitivity of the anti-HER2-Renilla luciferase fusion protein in cell experiments

To investigate whether anti-HER2-RLuc retained both HER2-binding and enzymatic light signal-producing activity, we first identified the HER2 protein expression level in two cell lines (**Figure 2A**). We then found a positive correlation between the light signal intensity and the number of SK-BR-3 cells. In contrast, MDA-MB-231 cells maintained a steady background light signal due to the absence of binding (**Figure 2B**). As anticipated, the bioluminescent signal of the fusion protein was gradually decreased in HER2-positive cells pretreated with a commercial anti-HER2 antibody in a dose-dependent manner (**Figure 2C**). Together, these data confirmed that anti-HER2-RLuc retains the epitope specificity of the parental antibody and specifically identifies the HER2 epitope expressed on cell membranes.

The detection and isolation of CTCs in breast cancer and many other cancers present a substantial technical challenge because these cells are rare. Therefore, the ability of this method to effectively detect these "rare" tumor cells is important. For this purpose, as shown in

Figure 2D, HER2$^+$ cells could be effectively detected by anti-HER2-RLuc among a large population of negative background cells even when the HER2$^+$ cell number was less than 10.

In vivo bioluminescence imaging with anti-HER2-luciferase fusion protein

Our study evaluated the tumor-targeting properties of anti-HER2-RLuc in athymic mice bearing subcutaneous xenografts of SK-BR-3 and MDA-MB-231 cells. In contrast to the results reported by Venisnik et al., who detected a signal in positive tumors as early as 2 h after injection of the Db-18-RLuc8 fusion protein, in our experiment, no signal was present at any time point after injection of the fusion protein [18]. As illustrated in **Figure 3A**, the mean tumor volumes (P2) of the two groups showed a large dynamic range, with a >2-fold increase in HER2$^+$ cell-inoculated mice over that of the HER2$^-$ cell-inoculated mice. In all four SK-BR-3-inoculated mice and in only one MDA-MB-231-inoculated mouse (P1), a visible neoplasm had formed by the end of the experimental period (**Figure 3B**). The HER2$^+$ CTC light signal in the HER2$^+$ cell-inoculated mouse blood was more than two-fold higher than that of the HER2$^-$ inoculated mice (**Figure 3C**).

scFv-luciferase enrichment method in BC patient blood samples

To determine whether this method could effectively detect HER2$^+$ CTCs in patient blood, we investigated its use in 59 peripheral blood samples from breast cancer patients. Of these, 12 were HER2 3+, 16 were HER2 2+, and 15 were HER2 1+ BC patients, whereas 16 and 14 were HER2$^-$ and breast fibroadenoma (BF) patients, respectively. The luminometry detection data in **Table 2** are expressed as the means ± SDs of the five groups. The light signal was analyzed, and differences with *p* values <0.05 were considered to show statistical significance between the HER2 3+/2+ breast cancer patients and the other three parallel groups. Each point in the scatter diagram represents one patient. Blood samples with HER2 3+ and 2+ scores by IHC had a significantly higher bioluminescence signal than the other three groups (**Figure 4A**).
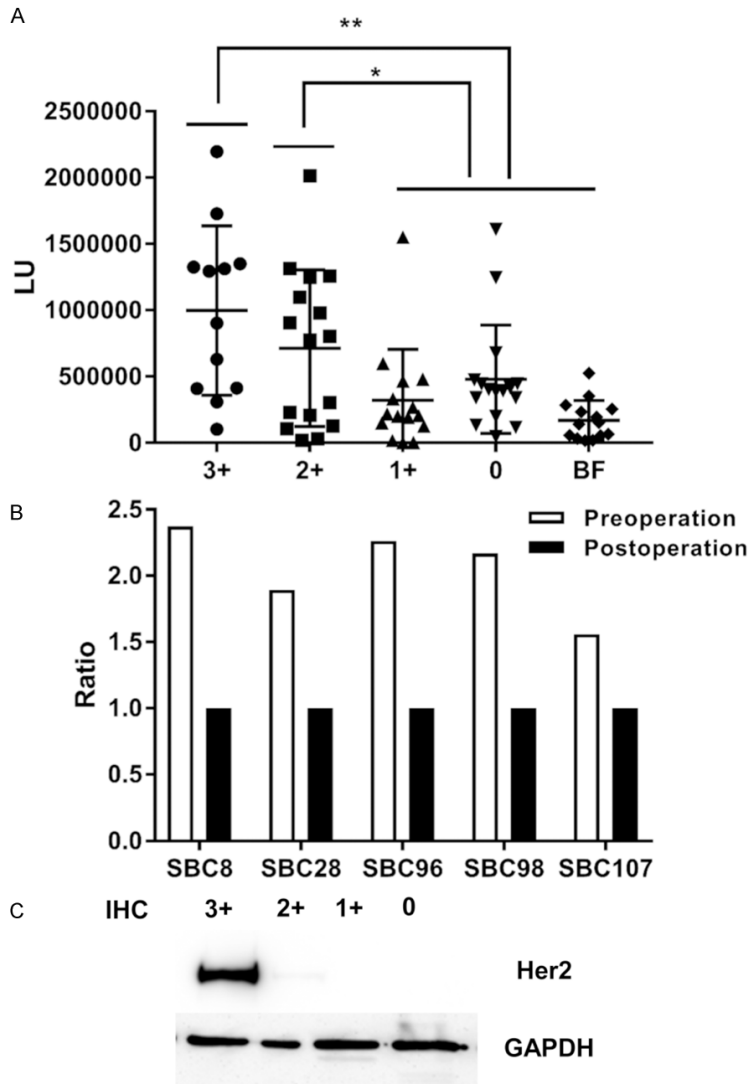
Figure 4. Application of the anti-HER2-luciferase fusion protein enrichment method in BC patient blood samples. A. The bioluminescence signal express detected in 59 BC patient samples with different HER2 scores by IHC. B. We selected five pairs of HER2 3+ patient blood specimens before and after surgery to exmie CTC HER2 levels. C. Western blot analysis was used to examine HER2 expression in the four different HER2 IHC phenotypes using patient tumor tissue specimens (n=4 groups).

Notably, we also found that three HER2 1+/- BC patients who were clinically diagnosed by IHC had high positive test results; we believe that this phenomenon is due, in large part, to the heterogeneity of cancer.

Five pairs of HER2 3+ preoperative and postoperative (one week after surgery) blood samples from breast cancer patients were also collected and evaluated in our study. As expected, the light signal intensity in all five groups exhibited varying degrees of decline after surgery com-

pared with the intensity in the preoperative samples (**Figure 4B**). We found a similar decrease in the HER2 2+ group and no clear difference between the preoperative and postoperative samples of the HER2 1+ and 0 groups (not shown).

Additionally, we performed western blotting analysis to further evaluate the HER2 score (0, 1+, 2+, or 3+) in the primary tumors tissues of BC patients (n=4 groups). As expected, we obtained the same results in all but one sample (1/4), and this tumor tissue, which was graded as HER2 3+, did not express HER2 protein (**Figure 4C**).

Screening CTCs by using protein A/G-coated plates

In this experiment, a similar increase in light signal intensity was found as an increasing number of SK-BR-3 cells was mixed with MDA-MB-231 cells (1×10^4) compared with MDA-MB-231 cells alone, in agreement with the results of previous experiments (**Figure 5A**). Immunofluorescence was used to confirm that our screened cells were HER2+ (**Figure 5B**).

To determine whether the protein A/G-coated plate screening method could effectively detect CTCs from breast cancer patients, we investigated its use in four pairs of peripheral blood samples, including three groups with different HER2 phenotypes and one HER2- group (**Figure 5C**). The results showed higher light signal intensity in the HER2 3+/2+ patients than in other groups of patients.

Detection of HER2+ exosomes from SK-BR-3 culture medium

Because exosomes are shed from nearly all cell types and often have similar membrane pro-
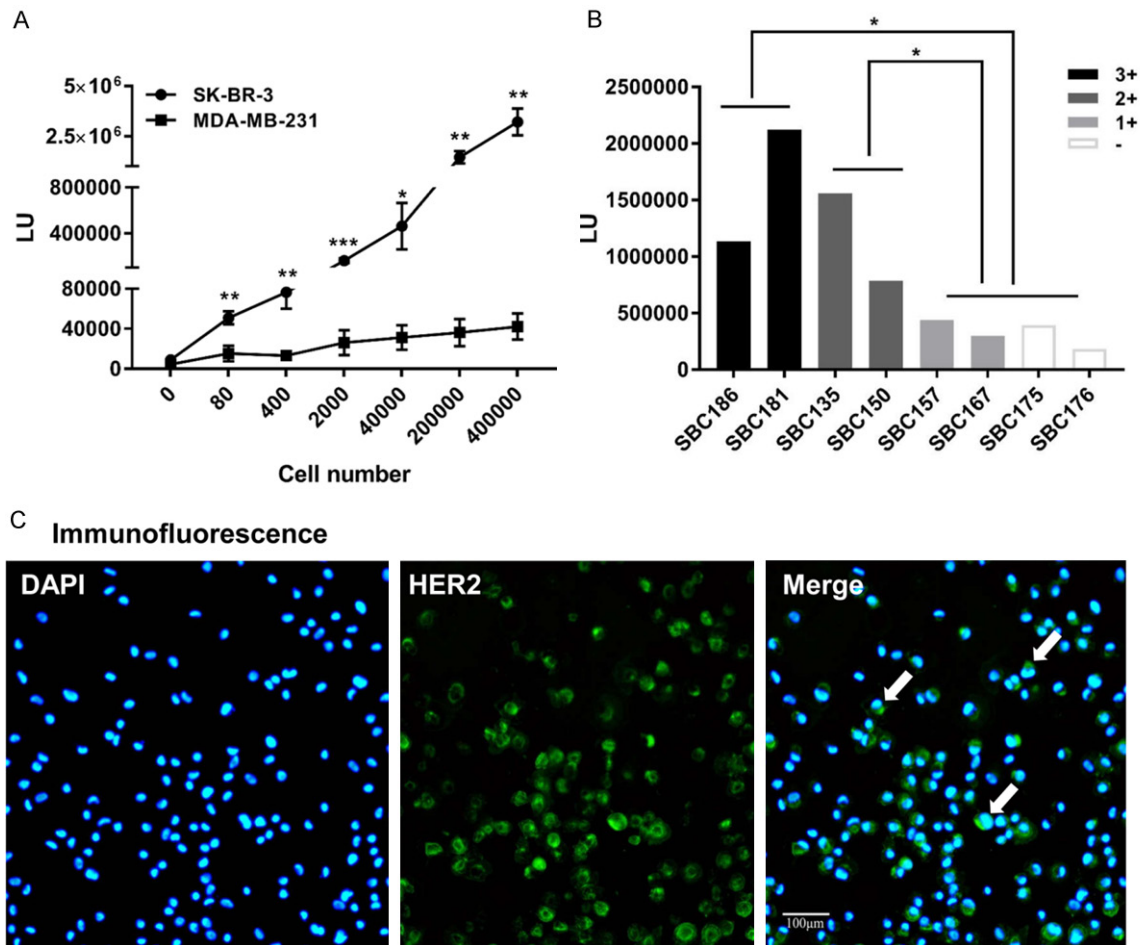
Figure 5. Screening of circulating tumor cells (CTCs) using protein A/G-coated plates. A. The diagram shows the light signal of the screened cells. To test the feasibility of this screening method, we incubated anti-HER2-RLuc (10^8 RLU) with MDA-MB-231 cells or with different numbers of SK-BR-3 cells mixed with a fixed number of MDA-MB-231 cells (10^4) and then transferred the cells to protein A/G-coated plates. After washing, the light signal was detected using an LB942 luminometer. B. Patient blood specimens were subjected to the same detection procedure (n=8). C. Immunofluorescence (IF) staining of screened HER2$^+$ CTCs.

teins as parental cells, we determined whether exosomes shed from SK-BR-3 cells in culture could be detected using the scFv-luciferase method. The K562 cell line, B cells, and complete RPMI 1640 culture medium were used as negative controls (note that unless removed, FBS will contain a variety of bovine-derived exosomes). Using the HER2 scFv-luciferase method, we detected a bioluminescent signal intensity that was 3-7-fold greater in the SK-BR-3 cells than in the control groups (**Figure 6A**). These results indicated that the HER2$^+$ exosomes shed by breast cancer cells might be a useful additional biomarker. To further elucidate the relationship between the volume of culture medium and the bioluminescent signal intensity, the indicated volume of culture media

was used for detection. However, no clear distinction was observed among the different volumes of culture medium, independent of the HER2$^+$ or HER2$^-$ status (**Figure 6B**).

Discussion

In the absence of clinically detectable overt metastases, CTC can provide unique and important information regarding the molecular characteristics of distant metastasis as a surrogate marker of minimal residual disease (MRD) [19]. Currently, various tumor markers such as CEA and CA15-3 are used to assess a patient's therapeutic response [20]. In recent years, however, CTC detection has demonstrated great potential as a prognostic and predic-
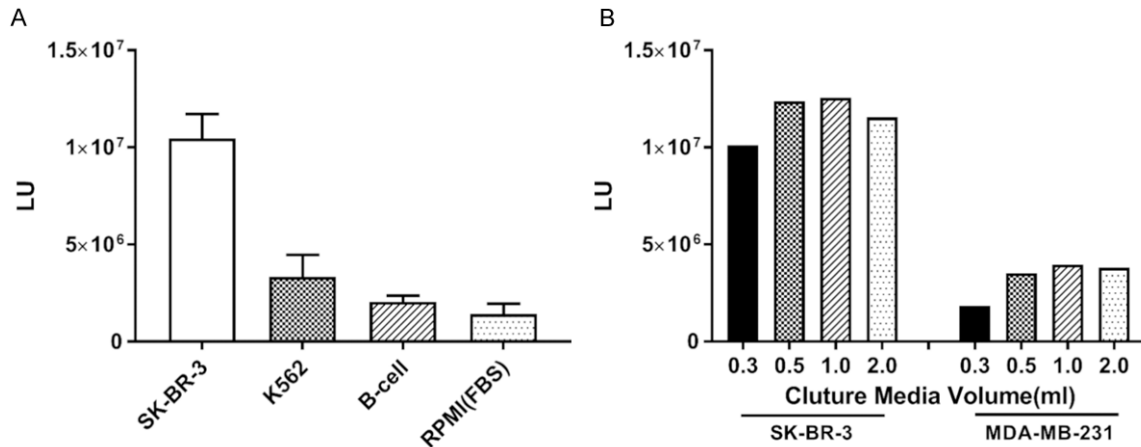
Figure 6. Exosome isolation and detection from culture media. All cells were cultured under standard conditions in RPMI 1640. A. One milliliter of culture media was collected and incubated with 5 μL of anti-HER2-RLuc for 1 hour at 4°C. Invitrogen exosome reagent was added (0.5 mL), and the mixture was incubated for 1 hour at 4°C and then centrifuged at 10,000 g for 10 min. The samples were washed three times, and the bioluminescence was then measured. B. The indicated volume of culture media was collected and subjected to the same procedure. The experiment was performed in triplicate.

tive tool through the expression of biological markers (such as HER2) in early-stage breast cancer [12, 21]; moreover, CTCs are likely superior to serum tumor markers [22]. HER2, which is encoded by the proto-oncogene ErbB2, is a member of the epidermal growth factor receptor family. This family contains tyrosine kinase activity receptors, which are mainly found on the cell surface of tumor cells as tumor-associated antigens [12, 23], and is involved in the regulation of cellular signaling during cellular growth and development [10, 18, 19]. Additionally, HER2 is recognized as an important predictive and prognostic marker and is a clinically relevant treatment target [23-25]. Considering the wide range of sensitivity (0.7-176 CTCs per mL of blood) and yields (14-100%) of CTC detection in patients with metastatic breast cancer, our aims in this study were as follows: (a) to determine whether scFv-luciferase-based technology can effectively detect HER2-positive CTCs in breast cancer with high sensitivity, and (b) to examine whether small populations of CTCs can be screened and distinguished from large populations of non-CTC cells.

In our study, we performed several experiments to assess whether this scFv-luciferase-based technology is able to detect CTCs in a large population of background cells. First, we conducted a series of *in vitro* cell experiments and confirmed the feasibility, specificity, and sensitivity of this detection method using two human breast cancer cell lines. The CTC detection method reported can easily detect HER2+ SK-BR-3 cells (**Figure 2B**). The HER2 expression level is an essential positive selection criterion in many CTC detection methods. Moreover, the anti-HER2-RLuc method exhibited high specificity and sensitivity and simultaneously recognized HER2+ antigens and generated a bioluminescent signal. Importantly, the anti-HER2-RLuc-mediated light signal of the SK-BR-3 cell line was significantly reduced when the cells were pretreated with different concentrations of HER2 antibody (**Figure 2C**), which illustrates the high specificity of the anti-HER2-Rluc fusion protein. In mixed-cell model experiments, we found a high detection sensitivity for SK-BR-3 tumor cells that were mixed with MDA-MB-231 cells. We designed three parallel groups to verify the sensitivity of this method, and anti-HER2-Rluc effectively distinguished HER2+ cells from HER2- cells, even if the number of positive cells in the reaction system was less than 10 (**Figure 2D**).

To ascertain the physical localization, tumors were induced in eight female athymic mice using the SK-BR-3 or MDA-MB-231 human breast cancer cell lines. Mouse tumor models were successfully established using these two primary tumor cell lines, which resulted in a

success rate of 62.5% (all four SK-BR-3 cell-inoculated mice but only one MDA-MB-231 cell-inoculated mouse formed a visible neoplasm). To a large extent, the success rate might be due to the degree of malignancy. The faster growth rate of HER2$^+$ tumors compared with that of the HER2$^-$ tumors is shown in **Figure 3A**. This result might indirectly indicate that HER2$^+$ breast cancer has a higher degree of malignancy. Following the bioluminescent imaging of tumor-bearing mice, we were surprised that anti-HER2-RLuc was unable to specifically target HER2$^+$ tumors and that no bioluminescent signal was detected in the living animals. However, Venisnik et al. demonstrated that light signals can be detected in CEA+ tumor-inoculated mice as early as 2 h after injection of fusion protein [18]. We suspect that the major cause of this phenomenon is associated with the instability of the fusion protein *in vivo* at 37°C (body temperature). Therefore, further studies are needed to evaluate the stability of the bioluminescence activity of the fusion protein in a 37°C environment. Nonetheless, we demonstrated the capability of anti-HER2-RLuc to localize preferentially to HER2-expressing tumors *in vitro*. Finally, whole blood was collected by heart puncture, assayed, and luminometry was performed. A significant difference was observed between these two types of tumors. As shown in **Figure 3B**, the light signal emitted by HER2$^+$ tumor-inoculated mice was more than two-fold higher greater compared with that emitted by HER2$^-$ tumor-inoculated mice.

The detection results from all patient blood samples revealed that 28 patients, 16 with a HER2 score of 2+ and 12 with a HER2 score of 3+, had a higher light signal than patients in the other three groups (**Figure 4A**). However, because tumors show biological heterogeneity, we found that some patients presented lower signal in these two groups, and three patients presented higher light signal in other three groups. Several studies have shown a high degree of discordance between the primary tumor and metastatic sites or CTCs with respect to hormone receptor and HER2 expression. Pestrin et al. demonstrated that some HER2$^-$ primary tumors might shed HER2$^+$ CTCs after multiple courses of therapy or during disease progression [13, 15]. These considerations suggest that the present results should be fur-

ther considered, and evaluation of HER2-targeted therapy in patients with HER2$^-$ primary tumors and HER2$^+$ CTCs is certainly important.

Based on the results of this study, we considered whether the expression level of HER2 on CTCs in peripheral blood showed differences before and after surgery in individual patients. In all the tested samples, the luminometry results showed varying degrees of decline after surgery (**Figure 4B**). These data further support the conclusions that the scFv-luciferase fusion protein method can effectively detect HER2-positive CTCs in breast cancer.

Immunohistochemistry (IHC), however, remains the most common method used for routine diagnostic evaluation of tumor tissues in clinical practice [26]. However, a drawback of IHC is its varied interpretations because it is a semi-quantitative test by nature and requires the subjective judgment of observers (Molecular Diagnosis of Breast Cancer). Therefore, it is of considerable interest to assess whether IHC can be used as a reliable reference standard in our research study. In accordance with the western blotting results, we found that both methods produced highly consistent results, although we only evaluated them in four different groups due to the tissue specimen limitations (**Figure 4C**).

In recent years, researchers have developed many technologies for CTC screening, including flow cytometry [27], CellSearch [28], CTC chip [29], and the CAM-capture method [30]. Based on these studies, we used protein A/G-coated plates to capture HER2$^+$ tumor cells. The light signal of the protein A/G-initiated CTC screening method reported here exhibited an effective ability to capture HER2$^+$ cells (**Figure 5A**). Specifically, the protein A/G-enriched SK-BR-3 tumor cells mixed with MDA-MB-231 cells were readily detected by the immunofluorescence (IF) assay (**Figure 5B**). In this case, we attempted to screen CTCs in four pairs of blood samples from breast cancer patients (3+, 2+, 1+ and 0). Similarly, as shown in **Figure 5C**, HER2 3+ BC patients exhibit an observably higher light signal compared with the other groups.

In addition to the successful detection of HER2$^+$ CTCs, to better characterize the effect of this detection method, we attempted to apply this strategy to the detection of HER2$^+$ exosomes.

Ciravolo et al. provided evidence that HER2$^+$ exosomes released either *in vitro* or in the serum of HER2-overexpressing breast cancer patients contain significant levels of activated HER2 [31]. These findings suggest that patients with advanced disease and high levels of circulating HER2$^+$ exosomes might be considered ideal candidates for HER2 detection using the anti-HER2-RLuc-based method. A 3- to 7-fold increase in the bioluminescent signal intensity was found in the HER2$^+$ BC cell-derived exosomes compared with the HER2$^-$ cell-derived exosomes.

A comparison of all the described *in vivo* and *in vitro* studies indicates that the advantages of our method over the current screening strategies are that it is simple, safe, rapid, inexpensive, quantitative, specific, and sensitive. The main limitations of our research study are as follows: (1) a lack of comparisons with healthy individuals, which implies the potential for confounding factors; (2) the limited number of patient blood and tissue specimens; and (3) the relatively higher level of fluorescence background, possibly due to a large quantity of leukocytes that were not removed from the blood. Future in-depth analyses of CTCs are needed to identify disease progression in patients with early-stage cancer.

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Disclosure of conflict of interest

None.

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References

[1] Pantel K, Brakenhoff RH and Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. Nat Rev Cancer 2008; 8: 329-340.

[2] Wang WJ, Lei YY, Mei JH and Wang CL. Recent Progress in HER2 Associated Breast Cancer. Asian Pac J Cancer Prev 2015; 16: 2591-2600.

[3] Scaltriti M, Nuciforo P, Bradbury I, Sperinde J, Agbor-Tarh D, Campbell C, Chenna A, Winslow J, Serra V, Parra JL, Prudkin L, Jimenez J, Aura C, Harbeck N, Pusztai L, Ellis C, Eidtmann H, Arribas J, Cortes J, de Azambuja E, Piccart M and Baselga J. High HER2 expression correlates with response to the combination of lapatinib and trastuzumab. Clin Cancer Res 2015; 21: 569-576.

[4] Barnes DM, Lammie GA, Millis RR, Gullick WL, Allen DS and Altman DG. An immunohistochemical evaluation of c-erbB-2 expression in human breast carcinoma. Br J Cancer 1988; 58: 448-452.

[5] Ping Z, Siegal GP, Harada S, Eltoum IE, Youssef M, Shen T, He J, Huang Y, Chen D, Li Y, Bland KI, Chang HR and Shen D. ERBB2 mutation is associated with a worse prognosis in patients with CDH1 altered invasive lobular cancer of the breast. Oncotarget 2016; 7: 80655-80663.

[6] Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW and Hayes DF. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004; 351: 781-791.

[7] Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, Doyle GV, Matera J, Allard WJ, Miller MC, Fritsche HA, Hortobagyi GN and Terstappen LW. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol 2005; 23: 1420-1430.

[8] Giuliano M, Giordano A, Jackson S, Hess KR, De Giorgi U, Mego M, Handy BC, Ueno NT, Alvarez RH, De Laurentiis M, De Placido S, Valero V, Hortobagyi GN, Reuben JM and Cristofanilli M. Circulating tumor cells as prognostic and predictive markers in metastatic breast cancer patients receiving first-line systemic treatment. Breast Cancer Res 2011; 13: R67.

[9] Janni WJ, Rack B, Terstappen LW, Pierga JY, Taran FA, Fehm T, Hall C, de Groot MR, Bidard FC, Friedl TW, Fasching PA, Brucker SY, Pantel K and Lucci A. Pooled Analysis of the Prognostic Relevance of Circulating Tumor Cells in Primary Breast Cancer. Clin Cancer Res 2016; 22: 2583-2593.

[10] Rack B, Schindlbeck C, Juckstock J, Andergassen U, Hepp P, Zwingers T, Friedl TW, Lorenz R, Tesch H, Fasching PA, Fehm T, Schneeweiss A, Lichtenegger W, Beckmann MW, Friese K, Pantel K, Janni W and Group SS. Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. J Natl Cancer Inst 2014; 106.

[11] Aoyama K, Kamio T, Nishikawa T and Kameoka S. A comparison of HER2/neu gene amplification and its protein overexpression between primary breast cancer and metastatic lymph nodes. Jpn J Clin Oncol 2010; 40: 613-619.

[12] Zidan J, Dashkovsky I, Stayerman C, Basher W, Cozacov C and Hadary A. Comparison of HER-2 overexpression in primary breast cancer and metastatic sites and its effect on biological targeting therapy of metastatic disease. Br J Cancer 2005; 93: 552-556.

[13] Jordan NV, Bardia A, Wittner BS, Benes C, Ligorio M, Zheng Y, Yu M, Sundaresan TK, Licausi JA, Desai R, O'Keefe RM, Ebright RY, Boukhali M, Sil S, Onozato ML, Iafrate AJ, Kapur R, Sgroi D, Ting DT, Toner M, Ramaswamy S, Haas W, Maheswaran S and Haber DA. HER2 expression identifies dynamic functional states within circulating breast cancer cells. Nature 2016; 537: 102-106.

[14] Meng S, Tripathy D, Shete S, Ashfaq R, Haley B, Perkins S, Beitsch P, Khan A, Euhus D, Osborne C, Frenkel E, Hoover S, Leitch M, Clifford E, Vitetta E, Morrison L, Herlyn D, Terstappen LW, Fleming T, Fehm T, Tucker T, Lane N, Wang J and Uhr J. HER-2 gene amplification can be acquired as breast cancer progresses. Proc Natl Acad Sci U S A 2004; 101: 9393-9398.

[15] Pestrin M, Bessi S, Galardi F, Truglia M, Biggeri A, Biagioni C, Cappadona S, Biganzoli L, Giannini A and Di Leo A. Correlation of HER2 status between primary tumors and corresponding circulating tumor cells in advanced breast cancer patients. Breast Cancer Res Treat 2009; 118: 523-530.

[16] Bard MP, Hegmans JP, Hemmes A, Luider TM, Willemsen R, Severijnen LA, van Meerbeeck JP, Burgers SA, Hoogsteden HC and Lambrecht BN. Proteomic analysis of exosomes isolated from human malignant pleural effusions. Am J Respir Cell Mol Biol 2004; 31: 114-121.

[17] Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G and Bonnerot C. Exosomal-like vesi-

cles are present in human blood plasma. Int Immunol 2005; 17: 879-887.

[18] Venisnik KM, Olafsen T, Loening AM, Iyer M, Gambhir SS and Wu AM. Bifunctional antibody-Renilla luciferase fusion protein for in vivo optical detection of tumors. Protein Eng Des Sel 2006; 19: 453-460.

[19] Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, Tejwani S, Schott AF, O'Rourke MA, Lew DL, Doyle GV, Gralow JR, Livingston RB and Hayes DF. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol 2014; 32: 3483-3489.

[20] Meller B, Rave-Franck M, Breunig C, Schirmer M, Baehre M, Nadrowitz R, Liersch T and Meller J. Novel carcinoembryonic-antigen-(CEA)-specific pretargeting system to assess tumor cell viability after irradiation of colorectal cancer cells. Strahlenther Onkol 2011; 187: 120-126.

[21] Gancberg D, Di Leo A, Cardoso F, Rouas G, Pedrocchi M, Paesmans M, Verhest A, Bernard-Marty C, Piccart MJ and Larsimont D. Comparison of HER-2 status between primary breast cancer and corresponding distant metastatic sites. Ann Oncol 2002; 13: 1036-1043.

[22] Bidard FC, Peeters DJ, Fehm T, Nolé F, Gisbert-Criado R, Mavroudis D, Grisanti S, Generali D, Garcia-Saenz JA, Stebbing J, Caldas C, Gazzaniga P, Manso L, Zamarchi R, de Lascoiti AF, De Mattos-Arruda L, Ignatiadis M, Lebofsky R, van Laere SJ, Meier-Stiegen F, Sandri MT, Vidal-Martinez J, Politaki E, Consoli F, Bottini A, Diaz-Rubio E, Krell J, Dawson SJ, Raimondi C, Rutten A, Janni W, Munzone E, Carañana V, Agelaki S, Almici C, Dirix L, Solomayer EF, Zorzino L, Johannes H, Reis-Filho JS, Pantel K, Pierga JY and Michiels S. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol 2014; 15: 406-414.

[23] Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A and McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 1987; 235: 177-182.

[24] Omenn GS, Guan Y and Menon R. A new class of protein cancer biomarker candidates: differentially expressed splice variants of ERBB2 (HER2/neu) and ERBB1 (EGFR) in breast cancer cell lines. J Proteomics 2014; 107: 103-112.

[25] Rakha EA, Pinder SE, Bartlett JM, Ibrahim M, Starczynski J, Carder PJ, Provenzano E, Hanby A, Hales S, Lee AH, Ellis IO; National Coordinating Committee for Breast Pathology. Updated UK Recommendations for HER2 as-

sessment in breast cancer. J Clin Pathol 2015; 68: 93-99.

[26] Dupouy DG, Ciftlik AT, Fiche M, Heintze D, Bisig B, de Leval L and Gijs MA. Continuous quantification of HER2 expression by microfluidic precision immunofluorescence estimates HER2 gene amplification in breast cancer. Sci Rep 2016; 6: 20277.

[27] Georgakoudi I, Solban N, Novak J, Rice WL, Wei X, Hasan T and Lin CP. *In vivo* flow cytometry: a new method for enumerating circulating cancer cells. Cancer Res 2004; 64: 5044-5047.

[28] Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AG, Uhr JW and Terstappen LW. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 2004; 10: 6897-6904.

[29] Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, Smith MR, Kwak EL, Digumarthy S, Muzikansky A, Ryan P, Balis UJ, Tompkins RG, Haber DA and Toner M. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 2007; 450: 1235-1239.

[30] Lu J, Fan T, Zhao Q, Zeng W, Zaslavsky E, Chen JJ, Frohman MA, Golightly MG, Madajewicz S and Chen WT. Isolation of circulating epithelial and tumor progenitor cells with an invasive phenotype from breast cancer patients. Int J Cancer 2010; 126: 669-683.

[31] Ciravolo V, Huber V, Ghedini GC, Venturelli E, Bianchi F, Campiglio M, Morelli D, Villa A, Della Mina P, Menard S, Filipazzi P, Rivoltini L, Tagliabue E and Pupa SM. Potential role of HER2-overexpressing exosomes in countering trastuzumab-based therapy. J Cell Physiol 2012; 227: 658-667.