Dynamic detection of HER2 of circulating tumor cells in patients with gastric carcinoma and its clinical application

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Abstract. The aim of the present study was to construct and characterize human epidermal growth factor receptor 2 (HER2) lipid magnetic ball (H-LMB) for separating circulating tumor cells (CTCs) in patients with gastric carcinoma (GC) and to compare the result of separated CTC counts with that of next-generation sequencing (NGS) for single-gene analysis to verify the consistency for evaluating the association between the detection results and the progress of clinical treatment, so as to facilitate early diagnosis and dynamic monitoring of GC. A lipid magnetic ball (LMB), coated with Fe_3O_4 nanoparticles, was synthesized by microemulsion technique and an anti-HER2 antibody was conjugated to the surface of LMB to form H-LMB, followed by the characterization of the prepared H-LMB. The detection of capture efficiency of LMBs in GC cells was tested by MTT and expression of HER2 mRNA was determined by reverse transcription-quantitative PCR. The positive detection rate of HER2 was verified by HER2-fluorescence *in situ* hybridization (FISH) test on the separated CTCs from GC. Further verification was performed based on the consistency between the result of separated CTCs and that of single-gene NGS assay of HER2, associated with the determination of clinical consistency. The constructed H-LMB exhibited good stability and specificity. The mutation rate of HER2 by the FISH test was 14% in the blood samples of 50 patients with GC and was 14% by NGS assay. The mutation rate of HER2 was 12% in H-LMB and the positive detection rate was 85.7% compared with the results of the FISH test, indicating consistency with the clinical diagnosis and pathological examination results. In conclusion, the anti-HER2 antibody-modified LMB can separate CTCs with HER2 abnormal expression, which exhibits an application potential in GC diagnosis and treatment and is of great clinical significance for the diagnosis and evaluation of its therapeutic effect on GC.

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Abbreviations: AFM, atomic force microscope; CEA, carcinoembryonic antigen; CTC, circulating tumor cells; DAPI, 4', 6-diamidino-2-phenylindole; DISH, dual-color in situ hybridization; EpCAM, epithelial cell adhesion molecule; FISH, fluorescence *in situ* hybridization; GC, gastric carcinoma; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; LMB, lipid magnetic ball; NGS, next-generation sequencing; PCR, polymerase chain reaction; ROC, receiver operating characteristic; TNM, tumor, nodes, metastasis; ToGA, Trastuzumab for Gastric Cancer; UV, ultraviolet

Key words: human epidermal growth factor receptor 2, lipid magnetic ball, circulating tumor cell, next-generation sequencing, gastric carcinoma

Introduction

Gastric carcinoma (GC) is defined as the primary epithelial cancer originating from the stomach. It ranks second in the world in terms of its incidence and mortality. There are nearly one million two hundred thousand newly diagnosed cases of GC reported annually worldwide and the incidence in China accounts for ~40% of all new cases (1-4). The widespread application of combined detection of serum biomarkers in clinical practice is to evaluate the therapeutic effect of solid tumors, but it is still limited due to its inadequate sensitivity and specificity (5,6). Circulating tumor cells (CTCs), derived from peripheral blood, exist from when the tumor begins to form, carrying the genetic information and changes in the tumor. They also have the advantages of noninvasive and convenient detection compared with tissue biopsy (7). The elucidation of genetic and phenotypic differences between primary and metastatic tumors may be facilitated on the basis of CTC analysis (8). The proportion of CTCs is relatively low in tumor cells, i.e., ~1-1,000 cells/10 ml, which are unable to be detected by traditional diagnostic methods (9). The present study applied a CTC separation and identification system with strong specificity and high capture efficiency and studied the

correlation between CTCs and clinical indicators in patients with GC and drug resistance.

Human epidermal growth factor receptor 2 (HER2), also known as c-erB2, belongs to the family of human epidermal growth factor receptors (10). The regulation of HER2 can occur at any level; gene, RNA or protein (11). Overexpression of HER2 has been reported in numerous types of cancer, including breast cancer, GC, ovarian cancer, endometrial cancer and lung cancer (12). Endoscopic biopsy is the primary choice for patients with advanced GC to obtain tumor tissue for HER2 evaluation. The positive rate of HER2 in GC is ~6%-30% and there is generally a poorer prognosis in patients with HER2-positive GC (13). However, one or more biopsies can provide limited information because of the high heterogeneity of HER2 expression along with certain side effects because of the invasive technique (14-19). The status of HER2 may change with the development of the disease or treatment process. It is, however, not feasible to evaluate HER2 by repeated evaluation of tissue in clinical practice (20,21). It is important to screen patients with anti-HER2-targeted therapy by detecting CTCs from peripheral blood so as to benefit the monitoring and management of its therapeutic effect on patients with GC.

Immunomagnetic separation of CTCs has been widely studied in lung cancer, colorectal cancer and breast cancer (22,23). Prior studies have focused on the comparison of the consistency of the separation of CTCs with HER2 abnormal expression from peripheral blood in patients with GC by anti-HER2 magnetic immunoliposomes with gold standard fluorescence *in situ* hybridization (FISH) for evaluating the status of HER2 (24,25). In addition to the inconsistency of the genomic map of primary with that of metastatic tumors, there is difficulty in obtaining tumor tissue clinically (26). Detection of circulating tumor DNA (ctDNA) by next-generation sequencing (NGS) provides an excellent choice for gene analysis and genotyping of patients with GC (27). In the present study, an anti-HER2 LMB was prepared to detect CTCs from patients with GC, to identify the subtypes of CTCs and to analyze the relationship with clinical characteristics of GC so as to achieve a dynamic detection and prognosis evaluation of patients with GC.

Materials and methods

Sampling. Samples collected and used consisted of peripheral blood samples of 50 patients with GC diagnosed by pathology and tumor tissues of 20 matched patients. Sampling was approved by the Ethics Committee of Zhabei District Central Hospital of Shanghai (approval no. ZBLL20180823) and patients signed informed consents. The HER2-positive GC cell line NCI-N87 was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, the Chinese Academy of Sciences.

Sample processing. Peripheral blood (7.5 ml) was collected from patients with GC in the hospital by medical anticoagulant blood collection tubes and the anticoagulant was EDTA-K2. The samples were stored at 4˚C to avoid freezing during storage, processing and transportation and were used within 72 h.

Detection index. Peripheral blood was collected for CTC count (magnetic separation immunofluorescence method was used for CTC count determination).

Clinical information collection. The clinical information of patients included their name (sample number), sex (32 male, 18 female), age (14 <65, 36 ≥65 years), clinical stage, pathology type and medication. The GC samples (peripheral blood and tumor tissue) were collected from Zhabei District Central Hospital from September 2019 to November 2020.

A key aim of the present study was to explore the dynamic monitoring of the CTC changes of the enrolled gastric cancer patients at least twice. The two time points set were preoperative (pre-treatment, when the patient was admitted to the hospital) and postoperative (post-treatment, one week after surgery).

Materials and instruments. RPMI medium, fetal bovine serum and trypsin were obtained from Gibco (Thermo Fisher Scientific, Inc.), anti-HER2 antibody (cat. no. ab134182) was from Abcam. PathVysion HER2 kit was from Abbott Pharmaceutical, magnetic liposomes materials were obtained from Huzhou Lieyuan Medical Laboratory Company Ltd.), the fluorescence microscope (BX61) was from Olympus Corporation, BI-90Plus laser particle size analyzer/Zeta potential analyzer and atomic force microscope (AFM) from Brookhaven Instruments Corporation and vibrating sample magnetometer (MPMS-XL-7) from Quantum Design, Inc.

HER2 mRNA in different gastric cancer cell lines expression level analysis. The Cancer Cell Line Encyclopedia (CCLE) database was employed to obtain HER2 mRNA expression in 38 gastric cancer cell lines (Fig. S1).

Cell transfection and HER2 expression detection. HER2 short interfering (si)RNAs were designed by Ambion online software and three siRNAs (HER2-siRNA-1, HER2-siRNA-2 and HER2-siRNA-3) were synthesized. The specific sequences were shown in Table SI.

Negative control (NC)-siRNA was the negative control. After extracting total RNA, cDNA was obtained according to the manufacturer's protocols instructions of the QuantiTect reverse transcription kit (cat. no. 205311; Qiagen GmbH). qPCR amplification was performed by the method provided in the instructions of the PCR detection kit (SYBR Premix Ex Taq II; cat. no. .RR820A; Takara). The relative quantitative method is $2^{-\Delta\Delta Cq}$ (28) and three independent experiments were performed.

RT-qPCR. The expression level of HER2 mRNA was detected in NCI-N87 cells transfected with HER2-siRNA-1, HER2-siRNA-2, HER2-siRNA-3 and NC-siRNA. The purity of RNA is 1.7<OD260/OD280<2.0 by spectrophotometer. The reaction conditions were pre-denaturation at 95˚C for 10 min and denaturation at 95˚C, then annealing at 55˚C for 15 sec and extending for 1 min for 32 cycles in total.

Preparation and characterization of LMB. The preparation steps of HER2 LMB (H-LMB) and EpCAM LMB (Ep-LMB) were as follows: Cholesterol, DOPC, GHDC and HQCMC were dissolved in dichloromethane and then Fe_3O_4 particles

were added. With a simultaneous addition of 0.1 mol/l PBS, the mixture was stirred and heated to 25˚C for 30 min to realize complete emulsification after shaking and mixing, which was the LMB. Furthermore, 0.6 mg anti-HER2 (anti-EpCAM) antibody was dissolved in 10 ml of isopropanol, supplemented with coupling agent EDC and NHS to mix with the prepared LMB, followed by stirring at 4˚C for 24 h at a constant speed, which was the anti-HER2 (anti-EpCAM) antibody-modified LMB, i.e., H-LMB.

During characterization, corresponding particle size and potential were measured following the dilution of 10 μl H-LMB (Ep-LMB) with 1 ml of distilled water. The prepared H-LMB (Ep-LMB) was observed using an atomic force microscope (AFM). An amount of 10 μl H-LMB (Ep-LMB) was taken and diluted with 1 ml of distilled water and then 50 μl of the diluted sample was smeared on the mica sheet for observation. The absorbance was measured at a wavelength of 280 nm with ultraviolet (UV) spectrophotometer with the dilution of another 10 μl H-LMB (Ep-LMB) with 1 ml of distilled water. The magnetic properties of H-LMB (Ep-LMB) and LMB were measured by a vibrating sample magnetometer. The method of obtaining Ep-LMB was the same as H-LMB.

Test for the capture ability of HER2-LMB to NCI-N87 gastric carcinoma cells. After counting, cultured GC cells with high expression of HER2 were divided into five groups at different cell count gradients of 10, 20, 50, 100 and 200 cells/ml. These were divided into H-LMB and EpCAM LMB (Ep-LMB) groups after dilution with 7.5 ml PBS solution, with three replicates in each group. These groups were used to test the ability of the prepared H-LMB to capture NCI-N87 GC cells and to compare with Ep-LMB. An amount of 20 μl H-LMB was added to 7.5 ml of sample, which was then incubated at room temperature for 20 min and mixed once after every 5 min. The centrifuge tube was then inserted into the Magnetic Separation Rack for 10 min (room temperature) and the PBS solution was added to wash twice after discarding the supernatant. Staining was performed in the dark for 20 min (room temperature) following the addition of 20 μl FITC-labeled CK19 antibody (CK19-FITC), 20 μl DAPI staining solution and 10 μl PE-labeled CD45 antibody (CD45-PE). Following staining another two times of washing with double distilled water were performed on the Magnetic Separation Rack to remove completely the unbound antibody and staining solution. In the final step, 20 μl double distilled water was added into the centrifuge tube to suspend again and evenly distributed on the polylysine-treated slide. Observation and counting were performed under a fluorescence microscope (10x magnification) after the liquid dried.

Immunohistochemistry (IHC). All specimens were processed by marking, sectioning, fixation and other steps within 30-40 min following collection. The tissues were fixed using 10% neutral formalin (room temperature for 2-12 h). All the samples were confirmed to be GC by medullary staining of a qualified pathological examination institution. The present study used the most representative specimens from gastroscope biopsy and the primary tumor specimens were resected surgically (postoperative surgical specimens). The specimens were paraffin-embedded, serially sectioned (5 μm), dewaxed with xylene and dehydrated using 100, 100, 95, 90, 80 and 70% ethanol (12-15 min each). The mouse anti-human HER2 monoclonal antibody (OriGene Technologies, Inc.) was used as the primary antibody. Immunohistochemical SP staining kit was purchased from OriGene Technologies, Inc. IHC was used to detect the protein expression of HER2 in three tissues. The positive control was provided by the Pathology Department and PBS was taken as the negative control for staining instead of the first antibody.

Test for the capture of circulating tumor cells by HER2-LMB and Ep-LMB from gastric carcinoma patients. The peripheral blood of 50 patients with GC and the tissues of 20 matched patients with GC were detected first by HER2-FISH test to calculate cases with HER2 abnormal expression. H-LMB and Ep-LMB were used to separate and screen CTCs from peripheral blood samples of 50 patients. Immunofluorescence identification and FISH were performed on the separated CTCs before and after treatment. Positive-CTCs were stained with -DAPI andCK19-FITC but not CD45-PE and exhibited typical tumor cell morphology (inconsistent size, shape and staining of nuclei). H-LMB and cells were stained with Prussian Blue Staining kit [nuclear fast red; cat. no. 60533ES20, San Yi Biotechnology (Shanghai) Co., Ltd.] for 10 min at room temperature.

Detection of HER2 in blood, tissue and circulating tumor cells of gastric carcinoma patients by FISH. The centromere of chromosome 17 (CEP17) was labeled green and the *HER2* gene was labeled red. Following heat and high-pressure treatment for 3 min, pepsin (0.2 mg/ml) digestion for 20-40 min, dehydration and drying, 10 μl probe mixture was added in the target area of tissue slice followed by slice covering and molding. The tissue slices were then placed into an *in situ* hybridization system, denatured at 83˚C for 5 min and hybridized at 45˚C overnight (14-18 h). With a rapid washing, the tissue sections were dried at room temperature, 15 μl DAPI solution added to the target area and sealed. The number of 100-cell colonies was counted after reaction in the dark for 20 min and the results were observed under the fluorescence microscope.

The relationship between HER2 expression and clinical characteristics of gastric cancer. The clinical characteristics of 50 patients with GC, such as age, tumor size, TNM stage and metastasis, were collected and analyzed in terms of their relationship with HER2 abnormal expression.

NGS. A set of sequencing primers (NGS Adapter Primer) were used to design by Sangon Biotech (sangon.com/), to sequence the exons and ±10 bp side introns of several related genes. DNA samples were used to prepare the captured target gene library and the Illumina high-throughput sequencing method (Novaseq, Hybrid selection, paired) was used for sequencing (detection by Genowise, genowise.com/). In the present study, some NGS detection results have been used as statistics, as shown in Table I. The sequencing data are available in NCBI Sequence Read Archive (ncbi.nlm.nih.gov/sra) with the accession number PRJNA767212.

Table I. Detection results of next-generation sequencing.

No.	Detection time	Targeted drug-related mutations with clinical significance		Clinical significance unclear variations		
		Gene name	Drug name	Gene name	Mutation/Amplification/ Fusion/Expression	Abundance (%)
1	20190421	ERBB2	Trastuzumab; not sensitive	NOTCH1	exon22 c.G3584A p.G1195E	6.69
				PIK3CD	exon6 c.G755T p.G252V	2.10
2	20180911	BRAF	cetuximab			
		KRAS	cetuximab			
		NRAS	cetuximab			
3	20190218	ERBB2	Trastuzumab; not sensitive	PRKDC	exon67 c.C9343A p.S3115Y	8.00
				PRKDC	exon67 c.G9351T p.D3118Y	5.54
				IRF2	exon3 c.G96T p.K32N	7.37
				ZNF217	exon1 c.G430T p.E144X	5.52
				KAT6A	exon17 c.A5090C p.Q1697P	4.46
				STAG2	exon17 c.C1537A p.L513I	3.85
				ERBB2	exon22 c.A2711T p.D904V	3.44
				FANCG	exon10 c.T1147G p.S383A	3.16
				DAXX	exon1 c.A52T p.S18C	2.44
				GNAS	exon1 c.C1172T p.P391L	1.98
				FOXL2	exon1 c.T743C p.L248P	1.85
				ARFRP1	exon7 c.G385C p.V129L	1.67
				MYC	exon2 c.G144C p.Q48H	0.96
				MST1	exon11 c.G1301A p.R434Q	0.87
				ARID1A	exon1 c.G562C p.G188R	0.79
				SMO	exon3 c.A671C p.E224A	0.61
				NSD3	exon21 c.G3623A p.R1208H	0.52
4	20190801	ERBB2	Trastuzumab/ fam-trastuzumab deruxtecan-nxki	SESN1	exon3 c.C294A p.F98L	2.35
				KDM6A	exon6 c.C514G p.R172G	1.12
				ARID1B	exon1 c.360_361delp.Q121Tfs*110	0.63
				ARID1B	exon1 c.363_366delp.Q121Hfs*5	0.63
5	20190707	BAP1	Nilaparib/olaparib/ olaparib + bevacizumab	KIT	exon16 c.G2281A p.E761K	2.70
		ERBB2	Trastuzumab/ fam-trastuzumab deruxtecan-nxki			
6	20190103	ERBB2	Trastuzumab/ fam-trastuzumab deruxtecan-nxki	BAP1	exon13 c.A1585C p.K529Q	1.31
				MEN1	exon3 c.T224G p.L75R	1.14
				CDK12	exon2 c.T1616G p.L539R	1.02
				SMAD4	exon3 c.250-1G>T	0.71

Statistical analysis. All data in the present study were analyzed by SPSS 20.0 statistical analysis software (IBM Corp.). Pairwise comparisons were performed using an un-paired Student's t-test. P=value of tumor stages (I/II, III and IV), T stage (T1/T2/T3/T4), N (N0/N1/N2/N3) and M (M0/M1) were calculated by Fisher' s test. the analysis of CTC numbers in pre-treatment group and post-treatment group was performed by a paired Student's t-test. The difference in the expressed level of Her2 among groups were calculated by ANOVA. Bonferroni was used for the post-hoc test used following ANOVA. All data were the results of three independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Preparation, characterization of H-LMB and flowchart of detection and analysis. Further research was focused on the separation and identification, quantitative statistics and gene detection of CTCs from peripheral blood of clinical samples. The detection technology is shown in Fig. 1.

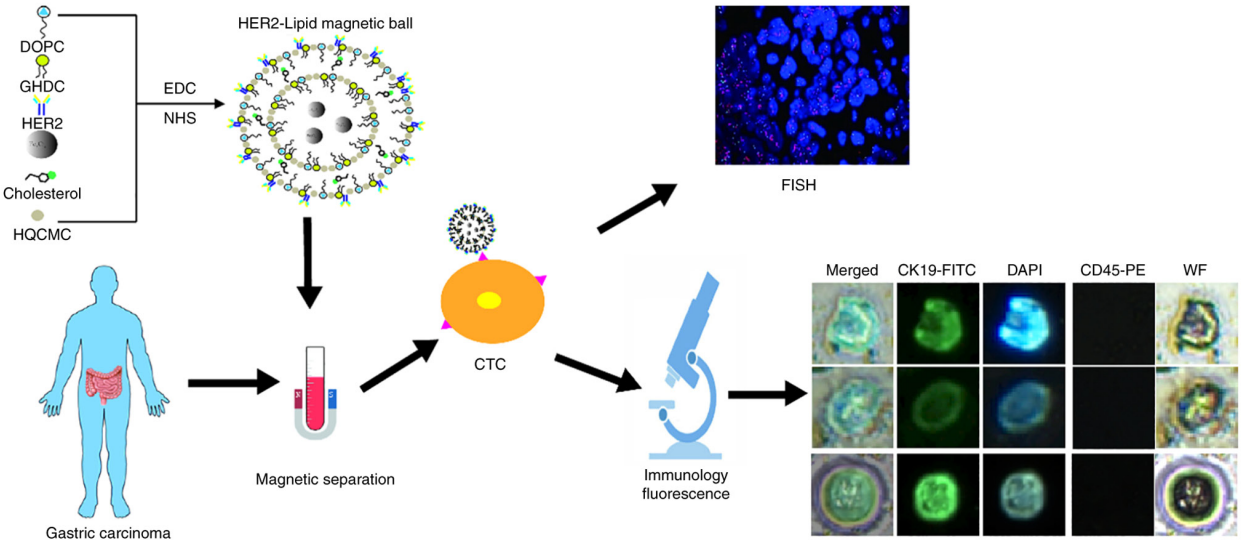

Figure 1. Preparation of HER2-lipid magnetic ball circulating tumor cell detection technology schematic. HER2, human epidermal growth factor receptor 2; CTC, circulating tumor cells; FISH, fluorescence *in situ* hybridization; WF, wide field

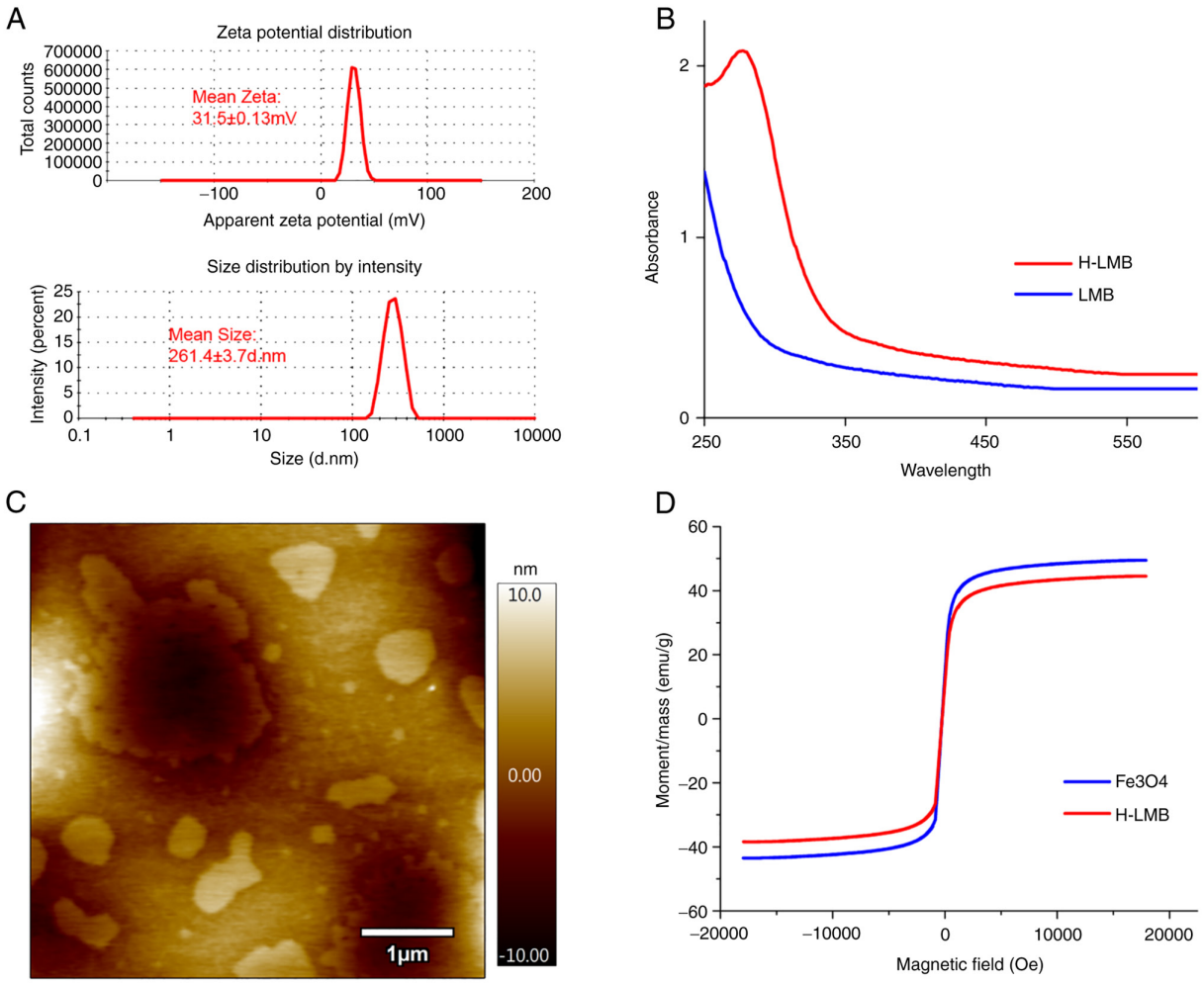

Figure 2. Characterization of HER2-LMB (H-LMB). (A) Potential distribution and particle size distribution of H-LMB. (B) Ultraviolet absorption spectra of H-LMB and LMB. (C) Atomic force image of H-LMB. (D) Hysteresis loop of H-LMB and Fe_3O_4. HER2, human epidermal growth factor receptor 2; LMB, lipid magnetic ball.

The preparation of LMB needed verification and the characterization of related indicators needed to be completed at the beginning of the experiment, as shown in Fig. 2. The charge of the synthesized magnetic lipid

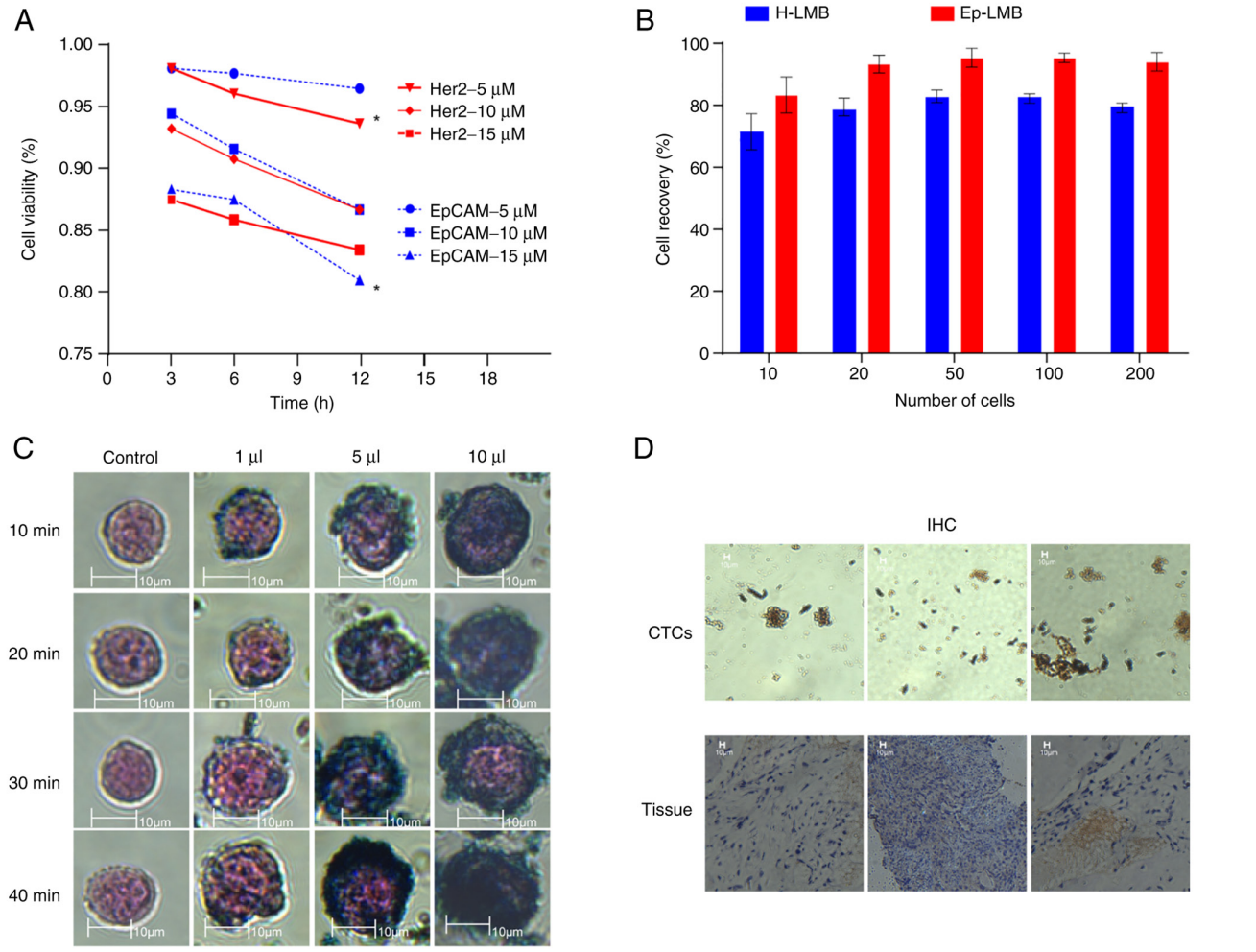

Figure 3. Cell line experiments. (A) Effects of HER2-LMB (H-LMB) and epithelial LMB (Ep-LMB) at different concentrations on cell proliferation. (B) Comparison of the capture ability of H-LMB and Ep-LMB at 10 μm. (C) Prussian blue staining of H-LMB in the cell line. (D) Immunohistochemical results of CTCs and tissue. *P<0.05. HER2, human epidermal growth factor receptor 2; LMB, lipid magnetic ball; CTCs, circulating tumor cells; IHC, immunohistochemistry.

nanoparticles was +31.5 mV; the average particle size was 261.4±3.7 nm distributed between 206.2 and 327.4 nm, showing a relatively narrow and uniform particle size distribution (Fig. 2A). The UV absorption spectrum showed that there was no absorption peak at 280 nm for LMB and a wide absorption peak at 280 nm for H-LMB, which were the properties of the protein. This suggested that the surface of the LMB was conjugated with an anti-HER2 antibody that was connected by EDS-NHS coupling reaction, exhibiting good specificity (Fig. 2B). AFM images revealed that the nanoparticles were spherical in shape with a size of ~250 nm with regular shape and without agglomeration. This was consistent with the particle size detection results (Fig. 2C). Fig. 2D shows the magnetic property test results of H-LMB. The hysteresis loop of H-LMB passed through the origin; besides, the coercive force and remanence were close to zero. This suggested that the prepared LMBs were superparamagnetic particles. It was concluded that the prepared nanoparticles were superparamagnetic LMBs modified with HER2 antibody.

Separation efficiency of H-LMB on gastric carcinoma cell line NCI-N87 and its interaction with cells. The relative expression levels of HER2 messenger RNA (mRNA) in 38 different gastric cancer cell lines are shown in Fig. S1A. HER2 overexpression in recombined plasmids (HER2 overexpression) and the empty vector (Vector) was detected by RT-qPCR (Fig. S1B). The results proved that the expression level of HER2 mRNA was downregulated in NCI-N87 cells following transfection with HER2-siRNA-1, HER2-siRNA-2 and HER2-siRNA-3, compared with that in NC-siRNA group. In addition, following stable transfer of the HER2 overexpression plasmid into NCI-N87 cells, the expression level of HER2 mRNA increased significantly; ~7 times higher than compared with the negative control group. With the increase of H-LMB concentration, the activity of gastric cancer cell line NCI-N87 decreased significantly (P<0.05).

The activity of cells below the cell concentration of 10 μM was still >90% after 6 h, which indicated good biocompatibility of H-LMB and Ep-LMB (Fig. 3A). Subsequent study focused on the experimental simulation of the capture ability of H-LMB for CTCs from NCI-N87 GC cells with HER2 abnormal expression to verify the separation effect on CTCs, as shown in Fig. 3B. The recovery rate of H-LMB to NCI-N87 GC cells was stable at different concentrations compared with Ep-LMB and the capture efficiency of H-LMB was >80%.

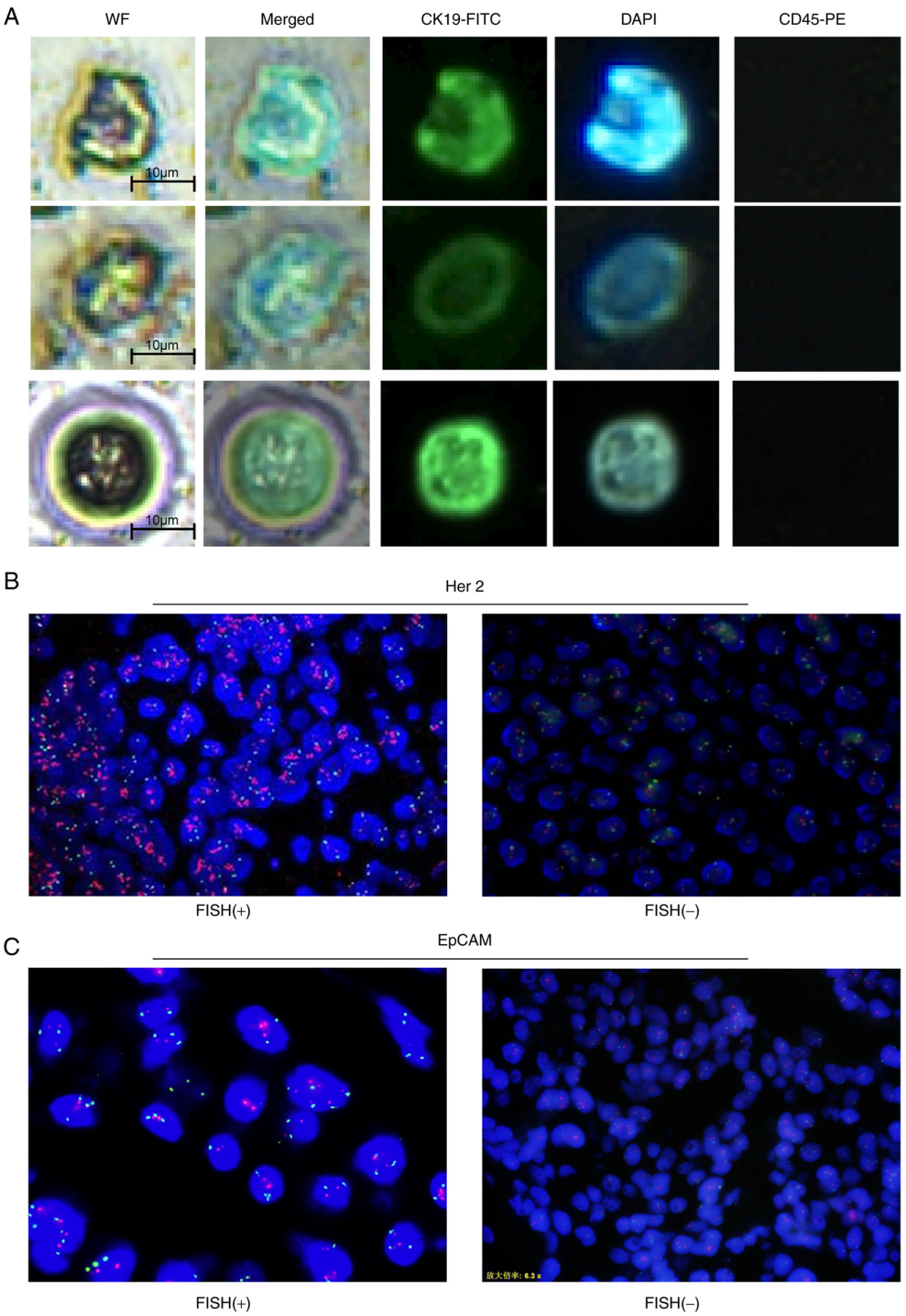

Figure 4. Clinical verification. (A) Immunofluorescence of CTCs from patients with gastric cancer captured by HER2-lipid magnetic ball. (B) Positive FISH results of the enriched CTCs. (C) Negative FISH results of the enriched CTCs. CTCs, circulating tumor cells; HER2, human epidermal growth factor receptor 2; FISH, fluorescence *in situ* hybridization; EpCAM, epithelial cell adhesion molecule; WF, wide field.

Therefore, H-LMB could be used to capture CTCs in clinical samples of GC with HER2 abnormal expression. The experimental results are shown in Fig. 3C. There was no blue signal in the blank control group, but it was observed on the cell surface after the addition of H-LMB. This suggested that H-LMB adsorbed on the cell surface and the signal intensity was positively correlated with incubation time and H-LMB concentration. The results of IHC showed that the staining of tissue was superior compared with CTCs (Fig. 3D). More detection results are shown in Fig. S2.

Clinical verification of circulating tumor cells. H-LMB and Ep-LMB were used to detect CTCs in blood samples of 50 patients with GC. Fig. 4A shows the immunofluorescence identification of CTCs captured by H-LMB. The CTCs in patients with GC had obvious cell morphology under the white light microscope, the green fluorescence of CK19-FITC was strongly positive and the blue fluorescence of DAPI was strongly positive, both of which overlapped after overlying. CD45 staining showed no fluorescence, which suggested that the captured cells were CTCs from GC.

Table II. Association between the positive rate of CTCs captured by H-LMB and tumor stage of GC patients.

Characteristic	n	H-LMB positive CTC, n (%)	H-LMB negative CTC, n (%)	χ^2	P-value	Fisher's test
Tumor stage				7.003	0.030[a]	0.031[a]
I/II	13	9 (69.23)	4.00 (30.77)			
III	6	6 (100)	0 (0)			
IV	14	14 (100)	0 (0.00)			
				7.031	0.071	0.084
T1	4	2 (50)	2 (50)			
T2	7	6 (85.71)	1 (14.29)			
T3	11	10 (90.9)	1 (9.1)			
T4	11	11 (100)	0 (0)			
				7.966	0.047	0.054
N0	12	8 (66.67)	4 (33.33)			
N1	12	12 (100)	0 (0)			
N2	8	8 (100)	0 (0)			
N3	1	1 (100)	0 (0)			
M				3.354	0.067	0.119
M0	19	15 (78.95)	4.00 (21.05)			
M1	14	14 (100)	0 (0)			

[a]P<0.05. CTC, circulating tumor cell; H-LMB, human epidermal growth factor receptor 2 lipid magnetic ball; GC, gastric carcinoma.

The region with the highest degree of amplification was selected for counting and calculation of the ratio of dichromatic signals of ≥20 consecutive tumor nuclei. When the ratio of total HER2 signals to total CEPl7 signals was ≥2.2, it was considered to be positive for *in situ* hybridization, i.e., amplification. When multiple signals were connected in clusters or the signal ratio of HER2 and CEPl7 was >20, the ratio could not be calculated and it could be judged as positive for *in situ* hybridization. When the ratio of total HER2 signals to total CEPl7 signals was <1.8, it was considered to be negative for *in situ* hybridization, i.e., no amplification. Typical cases are shown in Fig. 4B and C. The representative positive and negative FISH detection of HER2 and EpCAM are displayed.

Detection of circulating tumor cells in 50 patients using H-LMB. The detection rate of CTCs using Ep-LMB and H-LMB was 100% (n=50) and 72% (n=36) among the 50 patients with GC who were enrolled, respectively. The results of counting are shown in Fig. 5A. The count of CTCs decreased significantly with respect to prognosis in comparison to the pre- and post-treatment results (Fig. 5B), with the statistical difference (P<0.01) indicating its significance in the dynamic monitoring.

Dynamic monitoring of gastric carcinoma patients by circulating tumor cell count. Table II shows that the prepared H-LMB effectively captured CTCs from GC. The positive rate of CTCs was 69.23% (9/13), 100% (6/6) and 100% (14/14) in patients with TNM stage I-II, III and IV, respectively, with obvious differences among the subgroups (P=0.03). The positive rate of CTCs was 66.67% (8/12), 100% (12/12), 100% (8/8) and 100% (1/1) in patients with stage N0, N1, N2 and N3 lymph node metastasis, respectively, showing statistical differences among all subgroups (P=0.047).

Comparison of sensitivity and specificity between circulating tumor cells and traditional serum tumor markers in the diagnosis of gastric cancer. FISH was performed to investigate the blood samples of the 50 patients with GC. A total of five cases with HER2 abnormal expressions were detected in the tissue of patients with GC, five in the CTCs separated by H-LMB and six in the blood samples. The HER2-positive CTCs separated by H-LMB were consistent with that of tissue (HER2 expression in tumor samples and paired CTCs were 71.4%, the analysis of HER2 in CTCs showed higher positivity compared with tumor tissues). The mutation rate of HER2 was 10% in both tissue and CTCs of all the patients, while the positive rate of FISH was 14% in the blood samples. The ratio of HER2 and FISH was calculated by using the alternative control probe for one patient with uncertain HER2 status and a false-positive result was determined (Fig. 6A). Considering pathological diagnosis results as the gold standard, the sensitivity and specificity of CTCs detection were 77.1 and 89.6% in cancer diagnosis, respectively, as shown in Fig. 6B, which were higher than those of traditional serum tumor markers of carcinoembryonic antigen (CEA; 69.9 and 77.1%, respectively) and PIVIKA (59.9 and 67.1%, respectively), showing statistically significant differences. The receiver operating characteristic (ROC) curve is shown in Fig. 6B. The area under the ROC curve of CTCs in the diagnosis of gastric cancer was 0.86 [95% confidence interval (CI); 0.777-0.952], which was higher compared with CEA and PIVIKA. ROC curve analysis showed that the diagnostic value of CTC in gastric cancer was higher compared with traditional serum tumor markers.

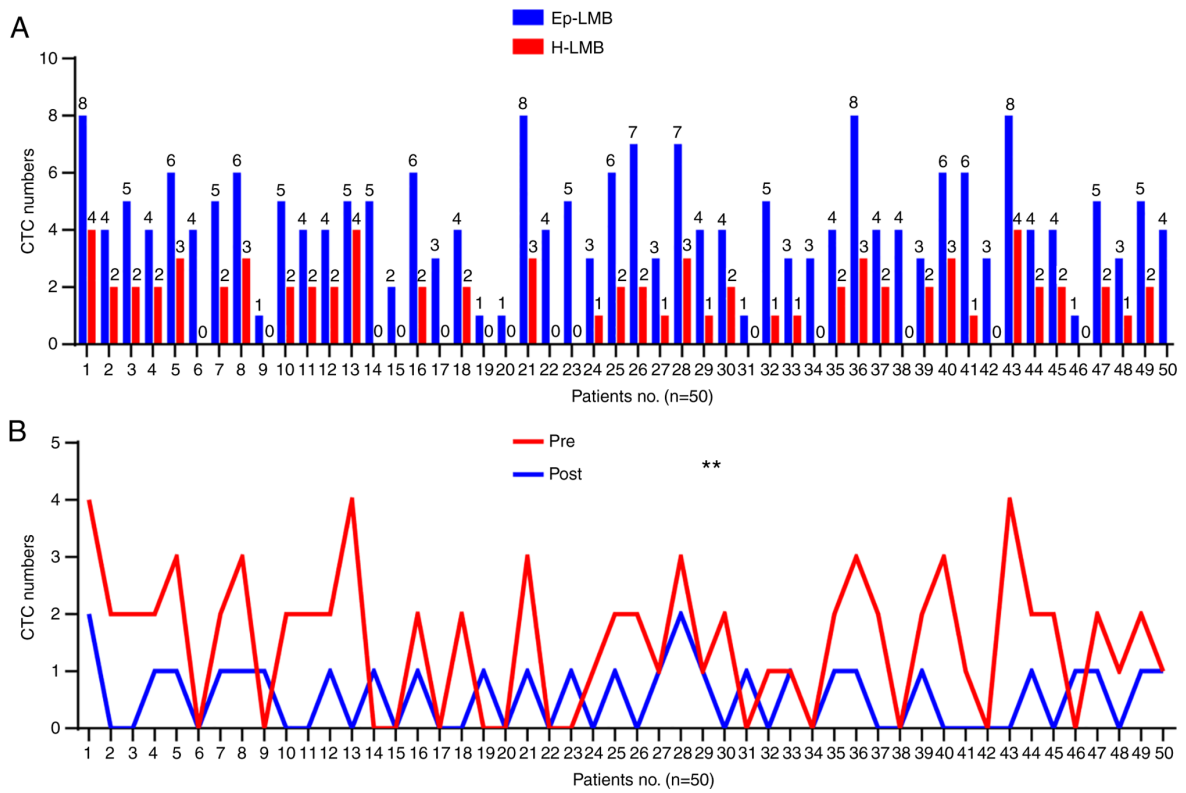

Figure 5. Counting CTCs. (A) The results of clinical CTCs with HER2-LMB (H-LMB) and epithelial LMB. (B) CTC results using H-LMB before and after treatment. **P<0.01 vs. pre-treatment. CTCs, circulating tumor cells; HER2, human epidermal growth factor receptor 2; LMB, lipid magnetic ball; Ep, epithelial; Pre, before; Post, after.

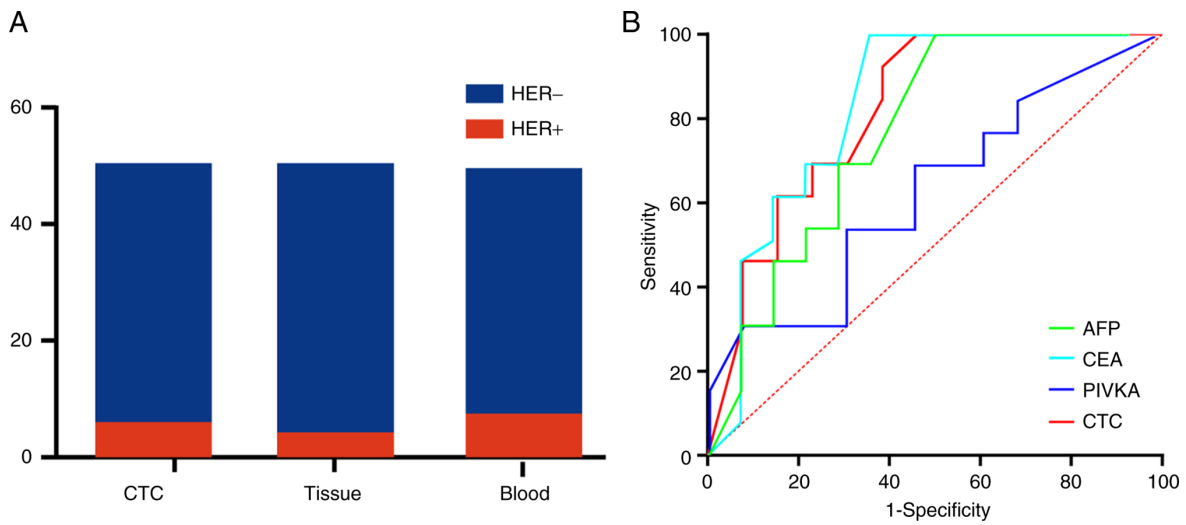

Figure 6. Comparison of CTCs. (A) Detection of HER2+ mutation from CTCs, tissue and blood. (B) Specificity and sensitivity of CTCs and other tumor markers. CTCs, circulating tumor cells; HER2, human epidermal growth factor receptor 2; AFP, α-fetoprotein; CEA, carcinoembryonic antigen; PIVKA, Protein Induced by Vitamin K Absence or Antagonist.

Discussion

GC has a strong heterogeneity. The traditional detection of serum tumor markers, such as CEA, carbohydrate antigen (CA)125 and CA199, frequently has lower specificity and sensitivity in GC and cannot meet the requirement of clinical application. Gastroscopy is considered as the gold standard for GC diagnosis, which, however, is ineffective for early GC screening and prognosis evaluation. It has been reported that a superficial infiltration of GC in the early stage may have an improved 5-year survival rate of 96-99% (29). CTCs can be detected when the tumor is 1 mm in size, which is difficult to achieve by conventional imaging technology. Moreover, most patients with GC are in the advanced stage on admission, with a 5-year survival rate of only 20-30% (30,31). CTCs can be detected throughout the whole process of cancer development

and exhibit an intimate correlation with the stage, metastasis, recurrence and prognosis of GC and their quantitative change has clinical significance (32).

HER2 is one of various molecular targets in clinical research with the most extensive application, in-depth research and explicit clinical significance (33). HER2-positive GC is one of the molecular subtypes of GC characterized by poor prognosis and shorter disease control duration of <6 months following standard chemotherapy. As confirmed by the ToGA study, trastuzumab combined with chemotherapy can significantly prolong the overall survival for >1 year in patients with HER2-positive metastatic GC (34). In this regard, it is of great significance for patients with GC to receive CTC dynamic monitoring combined with HER2 detection (20,21). The present study focused on the dynamic analysis of CTCs in GC. The detection of tissue samples was mainly used for comparison with peripheral blood samples. The purpose of detecting CTCs is improved evaluation of the rationality and efficacy of the treatment plan implemented on the patient before and after surgery or before and after radiotherapy and chemotherapy (35). As CTC-NGS is a dynamic multiple tracking test, the number and location of mutant genes obtained will be more based on CTC-derived genetic testing. The present study showed that CTC-DNA detection can be a good supplement to tissue testing and provide more testing resources for those patients from whom it is not easy to obtain tissue samples. It is impossible to evaluate the clonal complexity of patients with multiple metastatic diseases by sampling only one site of the disease due to the complexity of biochemistry and the dynamics of drug resistance (36). A previous study compared the copy number of the *HER2* gene in ctDNA from patients with pathological results (37). The detection of HER2 expression in plasma ctDNA was highly consistent with the IHC/dual-color *in situ* hybridization (DISH) method and the consistency was 91.07% (38). Gene detection by using NGS may be conducive to the clarification of the molecular mechanism of resistance to anti-HER2-targeted therapy. In the present study, CTC separation from the peripheral blood in patients with GC was performed by the preparation of H-LMB to screen CTCs with HER2 abnormal expression. The positive detection rate of HER2 was 12% and the consistency was 85.7% compared with that of FISH. Together, CTC detection is convenient and fast in operation and the screening of CTCs with HER2 abnormal expression can be used for dynamic detection in patients with GC and the development of an individualized therapeutic protocol. Of course, the present study also had some shortcomings: only the verification study was performed in the NCI-N87 cell line, other cell lines were not tested and more functional-related verification is required.

To sum up, the present study achieved the successful preparation of H-LMB with uniform size and surface antibody modification as a dynamic CTC detection platform for patients with GC. The prepared nanoparticles were superparamagnetic LMB modified with anti-HER2 antibody supported by LMB characterization for separation effect analysis of CTCs. It should be noted that the cells on which immunofluorescence was performed were dry cells and the cells were in a shrunken state. The images captured under the microscope are flat rather than three-dimensional, so CK19 and DAPI look like co-developed colors, but they are actually expressed in different positions. In addition, the experimental results of Chen *et al* (39) were consistent with the present study. It can be seen that this method is feasible and reproducible. With the application of NGS, changes at the gene and molecular levels are integrated based on the present study of dynamic changes in CTC counts from GC samples. The consistency analysis of HER2 abnormal expression in blood and tissue samples by FISH is of significance and clinical value for the systematic study of the influence and key role of HER2 in the development of GC.

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Availability of data and materials

All data generated and analyzed during the present study are included in this published article.

Authors' contributions

All authors participated in drafting and revising the manuscript critically for important intellectual content. MZ and Chunjin H designed the study. HZ, Chunyan H and ML collected and analyzed the data. Chunyan H and ML confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Zhabei District Central Hospital of Shanghai and participants signed informed consents (approval no. ZBLL20180823).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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