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ORIGINAL RESEARCH

Construction and Characterization of KRAS Immune Lipid Magnetic Balls for Colorectal Cancer Circulating Tumor Cells

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Objective: The purpose of this study was to prepare and characterize a lipid magnetic ball modified with KRAS antibodies on the surface and to isolate circulating tumor cells of colorectal cancer with *KRAS* mutations.

Methods: The microemulsion method was used to form lipid bilayers to encapsulate Fe3O4 nanoparticles with superparamagnetism to form lipid magnetic balls, and *KRAS* antibodies were formed on the surface to form *KRAS* immune lipid magnetic balls.

Results: Compared with traditional EpCAM antibody-modified lipid magnetic balls, it can effectively improve the capture ability of colorectal cancer circulating tumor cells with *KRAS* mutation, the capture rate reaches 92.9%, and the capture results are consistent with clinical diagnosis and pathology.

Conclusion: Our results showed that *KRAS* antibody-modified lipid magnetic balls can be used in the diagnosis and treatment of *KRAS* colorectal cancer.

Keywords: colorectal cancer, circulating tumor cells, *KRAS* mutation, lipid magnetic balls

Introduction

The incidence of colorectal cancer (CRC) is second only to lung cancer and breast cancer worldwide, accounting for about 10% of the total cancer worldwide.[1,2] Colorectal cancer is occult and the main cause of death is recurrence and metastasis. The overall 5-year survival rate is 50% −60%.[3,4] Circulating tumor cell (CTC) refers to tumor cells that fall off from the primary or metastatic foci into the peripheral blood circulation. CTC carries the genetic and phenotypic information of tumor tissues, which can guide patients with CRC to take drugs and detect resistance.[5,6] At present, the detection and research of CTC have been verified in colorectal cancer, non-small cell lung cancer, breast cancer and so on.[7–9]

KRAS gene (Kirsten rat sarcoma viral oncogene) participates in the regulation of signal pathways such as MAPK, PI3K and Ral-GEFs.[10–12] They are commonly found in digestive malignant tumors such as colorectal cancer, pancreatic cancer, and bile duct cancer,[13–15] and among non-digestive system malignancies such as endometrial cancer, breast cancer, lung cancer,[16–18] they are closely related to the occurrence, development, invasion and metastasis of these tumors. The occurrence of *KRAS* mutation is a negative predictor of CRC patients' drugs targeting *EGFR* (epidermal growth factor receptor).[19,20] Yang et al's research shows that *KRAS*

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mutations can be detected in CTCs of CRC patients, and 30% of patients without mutations have secondary *KRAS* mutations during the course of targeted therapy.[21]

Magnetic activated cell sorting (MACS) method is currently the most widely used and mature CTC capture method. Most of them are lipid magnetic balls modified with epithelial cell adhesion molecule (EpCAM) antibodies to isolate CTCs, but they are not ideal for CTC capture through epithelial–mesenchymal transition.[22,23] This study uses *KRAS* antibody-modified lipid magnetic balls to perform CTC detection on CRC patients, which can screen patients with *KRAS* mutations and better guide medications for CRC patients. Compared with traditional EpCAM lipid magnetic balls, more advantages in CRC.

Materials and Methods

Sample Collection

Peripheral blood was collected from 55 patients with pathologically diagnosed colorectal cancer in the Second Hospital of Baoding, Hebei Province. Of these, tissue samples of 15 patients were collected at the same time. The enrolled patients did not receive any adjuvant therapy. This study was approved by the Ethics Committee of the Second Hospital of Baoding, Hebei Province, and informed consent of each research participant was obtained, and an informed consent was signed in accordance with the "Helsinki Declaration". LoVo human colorectal cancer *KRAS* mutant cell line was purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Materials and Instruments

F-12K broth, fetal calf serum, trypsin were purchased from Gibco, 1-ethyl-3-(3-dimethylammoniumpropyl) carbonate (EDC), N-hydroxysuccinimide (NHS), Cholesterol, and other commonly used chemical reagents were purchased from Sinopharm, EpCAM lipid magnetic balls, Fe_3O_4, carboxymethyl chitosan hexadecyl quaternary ammonium salt (HQCMC), 1.2-dioleylphosphatidylcholine (DOPC), dimethyl octadecyl epoxypropyl ammonium chloride (GHDC) were purchased from Huzhou Liyuan Medical Laboratory Co., Ltd., *KRAS* gene upstream and downstream primers and PCR kit (B639289-0100) were purchased from Shanghai Sangon Biotech Co., Ltd., *KRAS* antibody (ab180772) was purchased from Abcam, BI-90Plus laser particle size analyzer/Zeta potentiometer was purchased from Brook-Haven Company, XL-30 environmental scanning electron microscope was purchased from PHILIPS, Netherlands, OLYMPUS B×61 fluorescence microscope was purchased from Olympus, Japan, and the MPMS-XL-7 vibration sample magnetometer was purchased from American Quantum Design Corporation.

Preparation of KRAS Lipid Magnetic Balls and EpCAM Lipid Magnetic Balls

KRAS-Lipid magnetic ball (K-LMB) and EpCAM-Lipid magnetic ball (Ep-LMB) were prepared according to reference.[24–26] Parallel experiments were carried out to select the appropriate antibody concentration and reagent concentration. Dissolve cholesterol, DOPC, GHDC, HQCMC, and Fe_3O_4 in dichloromethane, and add a 0.1 mol/L PBS solution at the same time. Mix the mixture by vigorous stirring and heat it to 25°C to completely emulsify it, forming the Lipid magnetic ball (LMB). Mechanical agitation is required during this period to avoid agglomeration or aggregation of iron oxide nanoparticles and nanocolloids. Take 0.6mg of *KRAS* antibody and dissolve it in 10mL of isopropanol. Add coupling agents EDC and NHS and mix with the nanomagnetic balls. Stir at 24°C for 24 hours to obtain *KRAS* antibody-modified lipid magnetic balls. The prepared lipid magnetic spheres were separated from the remaining solution and stored by magnetic separation.

K-LMB Characterization

Take 10μL K-LMB sample and dilute it with 1mL distilled water, then use BI-90Plus laser particle size analyzer/Zeta potentiometer to measure particle size and potential. To observe K-LMB with atomic force microscope (AFM) and aggregation occurred or not. Dilute 10μL K-LMB sample with 1mL distilled water, take 50 μL and coat it on the mica tablet, use non-contact tap mode (frequency = 325 Hz), scan at 1.0 Hz and observe after drying. Dilute 10 μL K-LMB and LMB samples in 1 mL of distilled water, measure the absorbance at 280 nm with an ultraviolet spectrophotometer. The magnetic properties of K-LMB were measured using a vibrating sample magnetometer.

K-LMB's Ability to Capture LoVo Colorectal Cancer Cells

The cultured LoVo colorectal cancer cells were added to the 7.5mL PBS solution at a gradient of 10, 20, 50, 100, 200 cells/mL after counting, and divided into K-LMB group and Ep-LMB group, each group has triplicate samples, the

prepared K-LMB was tested for its ability to capture LoVo colorectal cancer cells. Add 20 μL of K-LMB to a 7.5 mL sample, and incubate at room temperature for 15 min, mix once every 5 min. Insert the centrifuge tube into a magnetic separator for 10 min, discard the supernatant, and add PBS solution for washing twice. Add 10 μL of FITC CK19 monoclonal antibody (CK19-FITC), 20 μL DAPI staining solution, 10 μL PE-labeled CD45 antibody (CD45-PE), mix and stain for 15 min in the dark. After the staining, wash twice with deionized water to wash off unbound antibody and DAPI. Magnetically separate for 5 min. Finally, 15 μL of deionized water was added to the centrifuge tube to resuspend the CTC, evenly coat the poly-L-lysine-treated anti-off slides. After the liquid drops are dried, observe and count under a fluorescence microscope.

K-LMB and Ep-LMB Capture CTC Tests for CRC Patients

First, the peripheral blood of 55 patients with CRC and the tissues of 15 patients with CRC were tested for *KRAS* gene, and the cases with *KRAS* mutation were counted. Then, K-LMB and Ep-LMB were used to perform CTC tests on peripheral blood samples of 35 patients, and the isolated CTCs were subjected to immunofluorescence identification and *KRAS* gene detection.

Detection of KRAS Mutations in CRC Patient Tissues and CTCs

The *KRAS* mutations in the CRC tissues and CTCs were detected by PCR. The upstream primers of *KRAS* were 5′-GGGGAGGGCTTTCTTTGTGTA-3′, and the downstream primers were 5′-GTCCTGAGCCTG TTTTGTGTC-3′. The reaction parameters were: pre-denaturation at 95°C for 6min; denaturation at 95°C for 10s, annealing at 55°C for 10s, and extension at 72°C for 30s, a total of 50 cycles. The experiment was repeated independently at least 3 times, and 3 replicates were set for each sample.

Statistical Analysis

All the data in this study were analyzed by SPSS 20.0 statistical analysis software. The comparison between the two groups was analyzed by independent sample *t* test. Statistical standards of $\alpha = 0.05$ and $P < 0.05$ were used to judge that the difference was statistically significant. All data are the result of 3 independent repeated experiments.

Results

We synthesized the required *KRAS* lipid magnetic balls and performed characterization and verification of the effect of separating CTC. The technical route is shown in Figure 1.

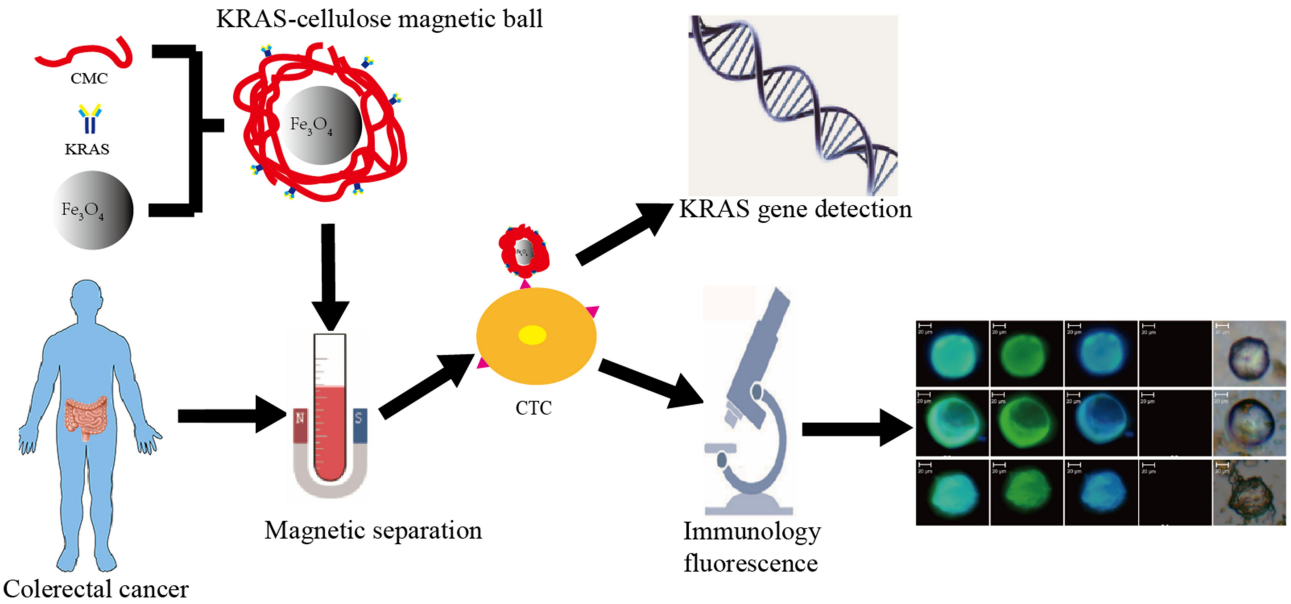

Figure 1 K-LMB preparation and CTC detection technology route.
Abbreviations: CTC, circulating tumor cell; K-LMB, *KRAS*-lipid magnetic ball.

K-LMB Characterization

A series of characterization tests were performed on K-LMB. Figure 2 shows the particle size and potential distribution, AFM image, and UV absorption spectrum of K-LMB. The synthesized lipid magnetic nanoparticles are spherical, with an average particle size of 198.3 ± 4.2 nm, and the distribution is between 110.1 and 356.2 nm. The particle size distribution is narrow and relatively uniform. The magnetic ball has a charge of +29.7 mV. The AFM image shows that the nanoparticles are spherical with a size of about 250 nm, and the shape is more regular. There is no agglomeration and the results are consistent with the particle size detection results. The ultraviolet absorption spectrum showed that the lipid magnetic sphere had no absorption peak at 280 nm, and K-LMB showed a broad absorption peak at 280 nm, which had protein characteristics, indicating

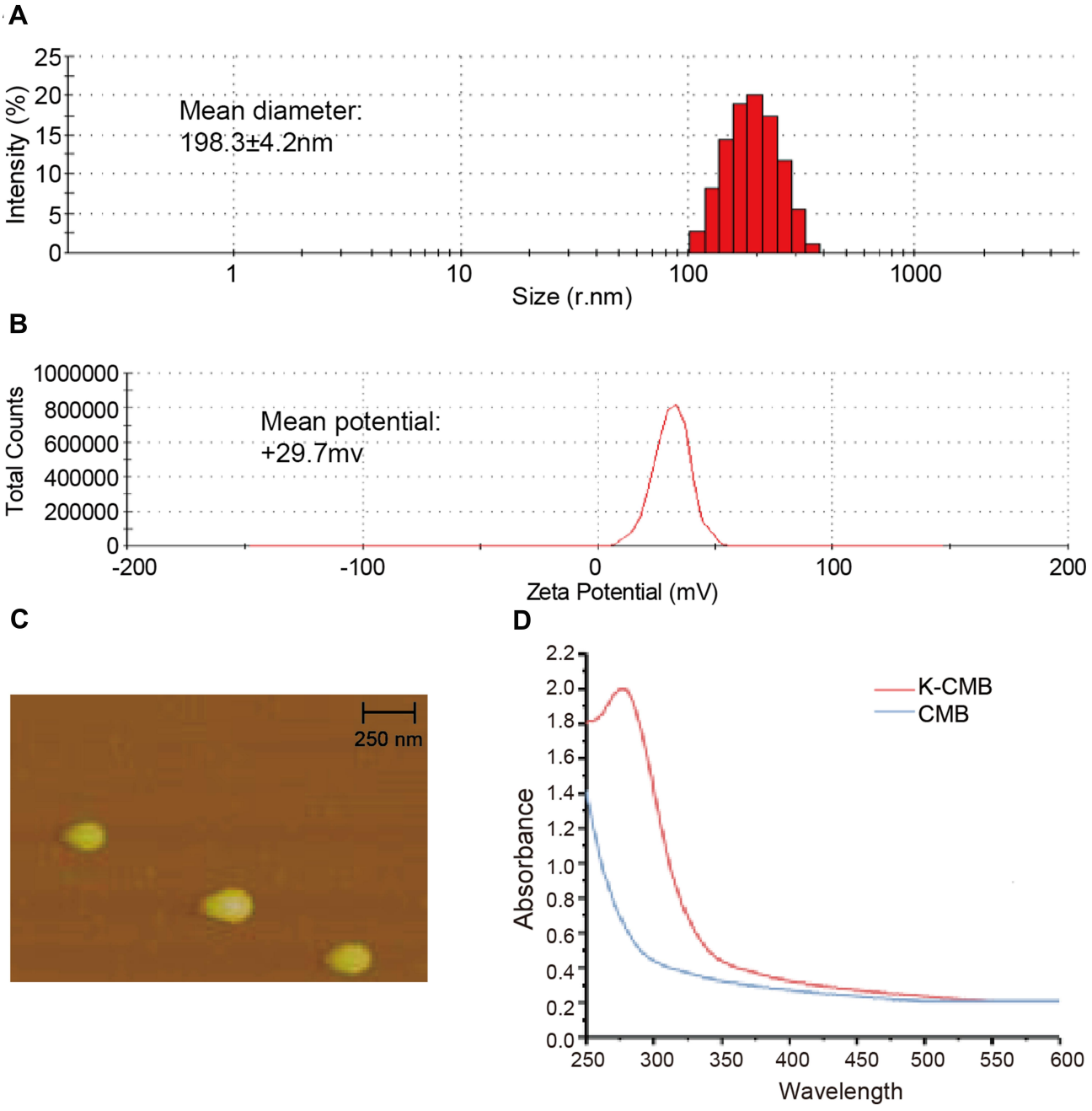

Figure 2 (**A**) The particle size distribution of K-LMB; (**B**) the potential distribution of K-LMB; (**C**) the atomic force image of K-LMB; (**D**) the ultraviolet absorption spectrum of K-LMB and LMB.

Abbreviation: K-LMB, *KRAS*-lipid magnetic ball.

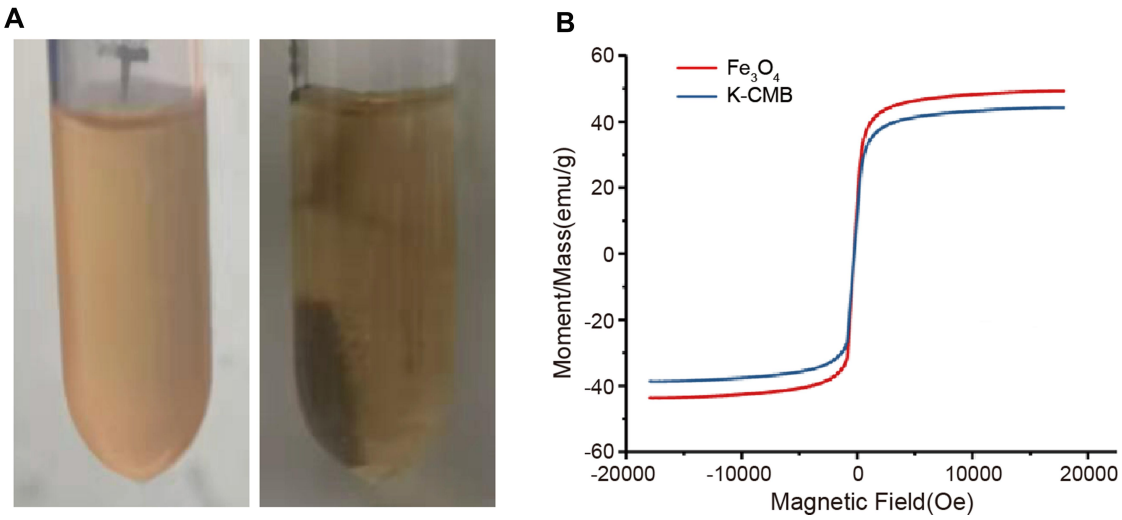

Figure 3 (**A**) The magnetic response of K-LMB under an external magnetic field; (**B**) the hysteresis loop of K-LMB.
Abbreviation: K-LMB, *KRAS*-lipid magnetic ball.

that the surface of the lipid magnetic sphere was connected by an EDS-NHS coupling reaction. *KRAS* antibody with good specificity. Figure 3 shows the results of the magnetic performance test of K-LMB. The hysteresis loop of K-LMB passed the origin, and the coercive force and remanence are close to zero. Under the action of an external magnetic field, K-LMB dispersed in the solution can be collected, and when the magnetic field disappears, K-LMB can be re-dispersed into the solution after shaking, which indicates that the prepared lipid magnetic balls are super-cis magnetic particle.

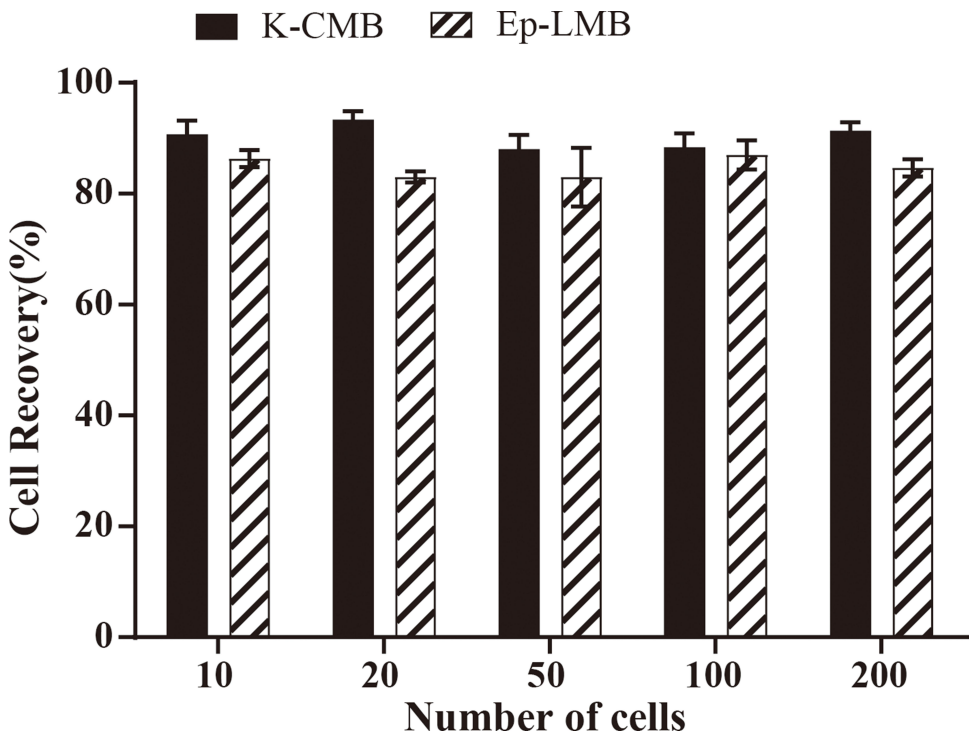

Figure 4 Capability of K-LMB and Ep-LMB to capture *KRAS* mutated LoVo colorectal cancer cells.
Abbreviations: Ep-LMB, EpCAM-lipid magnetic ball; K-LMB, *KRAS*-lipid magnetic ball.

Capability of K-LMB to Capture LoVo Colorectal Cancer Cells

Using the prepared K-LMB to capture the *KRAS* mutation of LoVo colorectal cancer cells, the experiment was performed to simulate the separation effect of CTC. The results are shown in Figure 4. Compared with Ep-LMB, the recovery of LoVo colorectal cancer cells by K-LMB is more stable at various concentrations. The capture efficiency of K-LMB is 90%, so it can be used for CTC capture of CRC clinical samples with *KRAS* mutations.

K-LMB Positive Rate of CTC in CRC Patients

K-LMB and Ep-LMB were used to perform CTC detection of the blood of 55 CRC patients. The immunofluorescence identification of CTC captured by K-LMB is shown in Figure 5. CTC of CRC patients has obvious cell morphology under white light, CK19-FITC green fluorescence is strongly positive, DAPI blue fluorescence is strongly positive, the two fluorescence overlaps after superposition, and CD45 staining does not show fluorescence. The captured cell is determined to be colorectal Cancer CTC.

The CTC isolation rate of 55 CRC patients enrolled was 100% (n = 55) using Ep-LMB and 54.5% (n = 30) using K-LMB. The counting results are shown in Figure 6.

The Effect of K-LMB on CTC Isolation in Patients with KRAS Mutant CRC and Its Relationship with Clinicopathological Characteristics

KRAS mutations were analyzed in the blood of 55 patients with CRC and in tissues of 15 patients with CRC. Among them, 26 patients had KRAS mutations in the blood and 5 patients had *KRAS* mutations in the tissues, and 16 patients had KRAS mutations detected in both tissues and blood. KRAS mutation detection was performed on 30 K-LMB isolated CTCs, and the results showed that the *KRAS* mutation rate was 100% (n = 30). In general, K-LMB detected 92.9% of CTCs with *KRAS* mutation in CRC patients. In order to clarify the correlation between *KRAS* mutations and other clinicopathological characteristics in CRC, the clinical and pathological characteristics of 55 patients with CRC were statistically analyzed, and important clinical parameters such as age, tumor size, lymph node metastasis, distant metastasis and TNM staging were selected for correlation,

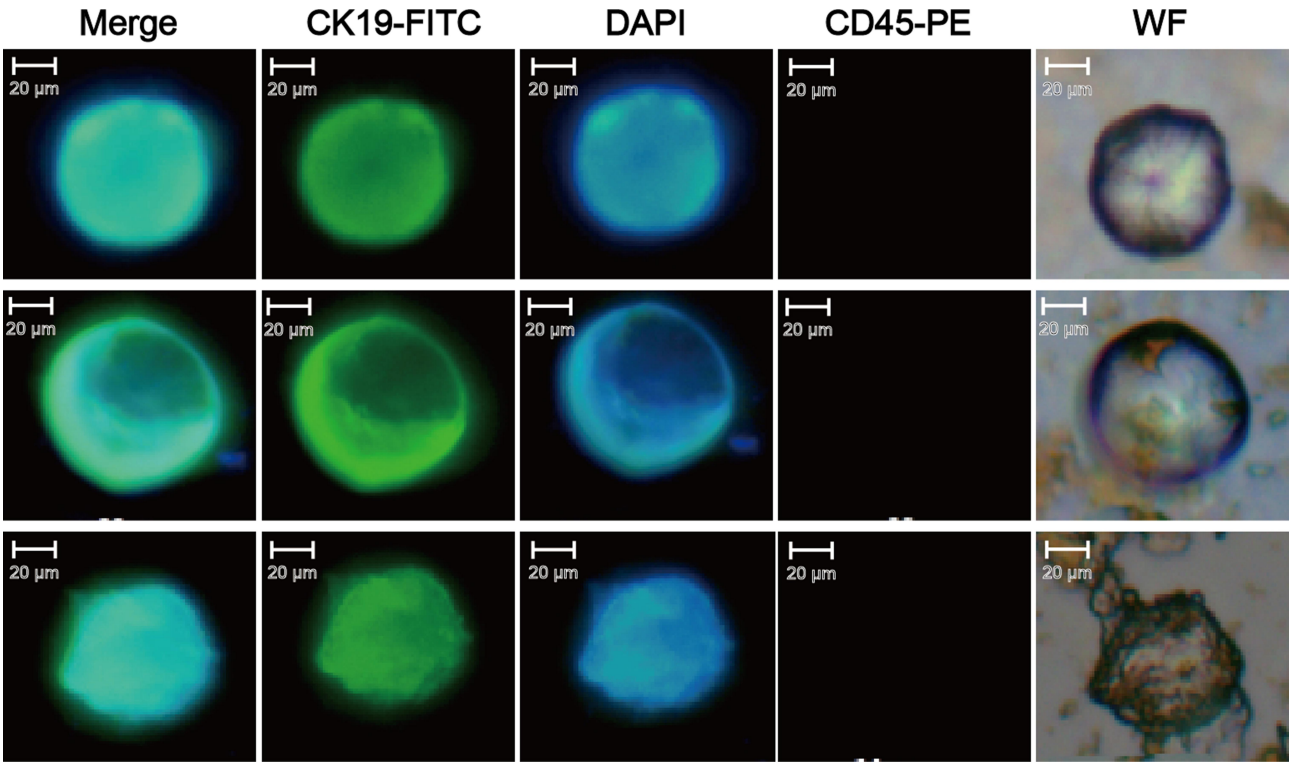

Figure 5 CTC immunofluorescence identification results of CRC patients captured by K-LMB.
Abbreviations: CRC, colorectal cancer; CTC, circulating tumor cell; K-LMB, *KRAS*-lipid magnetic ball.

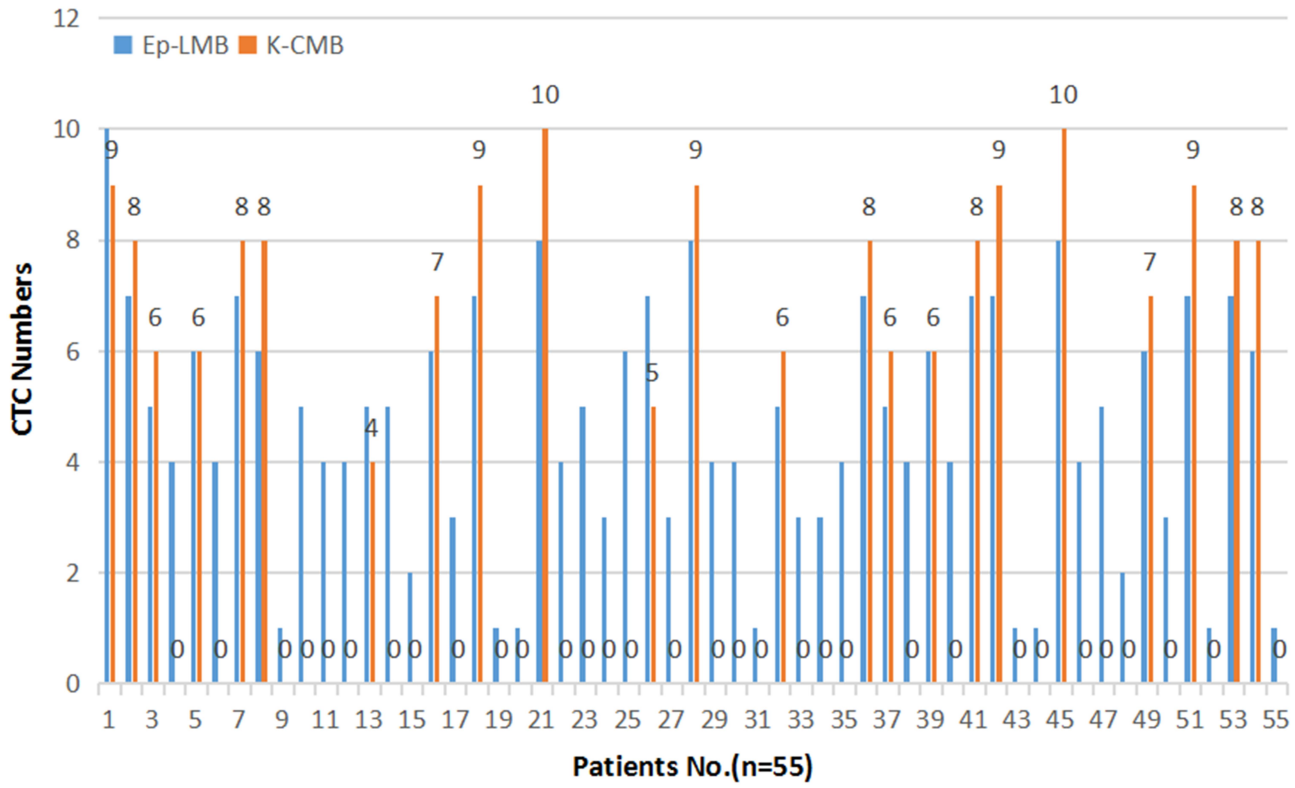

Figure 6 CTC test results of 55 CRC patients using K-LMB and Ep-LMB, respectively.
Abbreviations: CRC, colorectal cancer; CTC, circulating tumor cell; Ep-LMB, EpCAM-lipid magnetic ball; K-LMB, *KRAS*-lipid magnetic ball.

correlation analysis is shown in Table 1. The results showed that *KRAS* mutation was not related to other clinicopathological features (P <0.05).

Discussion

Liquid biopsy has become a hot spot in current cancer treatment, with more and more new technologies and methods having benefited patients with colorectal cancer. Immune nanomagnetic balls have been widely used and studied in colorectal cancer due to their unique advantages.[27] Compared with the traditional EpCAM lipid magnetic spheres, the lipid magnetic spheres with *KRAS* antibodies prepared by our research can screen patients with *KRAS* mutations in CRC, and the positive detection rate is high. The prepared KRAS lipid magnetic balls have uniform specifications, stable properties and high specificity. They are ideal magnetic balls for CTC separation of CRC patients.

The membrane-like structure of the lipid magnetic ball minimizes cell damage during CTC separation. The use of *KRAS* antibody can better personalize the *EGFR*-targeted therapy of CRC patients before and after drug resistance and improve detection Specificity. There have been studies on the preparation of *EGFR* antibody-modified lipid magnetic balls for CTC detection of CRC and detection of *KRAS* mutations, with a *KRAS* mutation rate of 71.4% (5/7).[28] The number of samples included in this study is too small and not representative. We used *KRAS* antibody-modified lipid magnetic balls to be more targeted.

At present, there have been studies on CTC screening for *KRAS* mutations in CRC. Taieb et al analyzed 3934 MSS stage III CRC patients found that the patients with *KRAS* mutations had a much shorter postoperative recurrence time, survival time after relapse, and overall survival time, and the overall survival risk with *KRAS* mutations is 1.62 compared with wild-type patients, indicating that *KRAS* mutations are independent predictors of postoperative recurrence, post-relapse survival, and overall survival deterioration in colorectal cancer.[29] In addition, some research tested the tumor tissue and CTC for *KRAS* mutations at the same time. The test results show that the consistency rate of the primary tumor tissue and CTC mutations can reach 70%.[30] The expression of EpCAM on CTC is very variable. The epithelial characteristics CTC undergoes interstitial transformation, making EpCAM magnetic balls

Table 1 Relationship Between *KRAS* Mutations and Clinical Characteristics in Blood from 55 CRC Patients and Tissues from 15 CRC Patients

	KRAS							
	Blood				**Tissue**			
Variables	Total n(n=55)	Yes (n=26)	No (n=29)	P-value	Total n(n=15)	Yes (n=5)	No (n=10)	P-value
Age, years								
<50	13	9	4	0.04	2	1	1	0.02
≥50	42	17	25	0.02	13	4	9	0.04
Tumor size								
T1-T2	15	6	9	0.05	2	1	1	0.02
T3-T4	40	18	22	0.03	13	4	9	0.04
Grade								
Well and moderately	17	8	9	0.015	2	1	1	0.02
Poor	38	16	22	0.04	13	4	9	0.04
Lymph node metastasis								
Present	25	12	13	0.04	2	1	1	0.02
Absent	30	12	18	0.04	13	4	9	0.04
Distant metastasis								
Present	24	13	11	0.035	2	1	1	0.02
Absent	31	13	18	0.04	13	4	9	0.04
TNM stage								
I-II	22	16	6	0.04	2	1	1	0.02
III-IV	33	13	20	0.025	13	4	9	0.04

Abbreviations: CRC, colorectal cancer; *KRAS*, Kirsten rat sarcoma viral oncogene; TNM stage, tumor node metastasis.

unsuitable for other tumors.[31] Our research can directly screen CTCs with *KRAS* mutations in patients with CRC, which can guide patients' medication before treatment, determine prognosis, and more accurately detect whether patients are suitable for targeted therapy.

In summary, the *KRAS* antibody-modified lipid magnetic balls prepared in this study are more suitable for CTC detection in CRC than EpCAM lipid magnetic balls, and it has been confirmed that *KRAS* mutations can be screened by CTC in the CRC in the past to achieve the possibility of early diagnosis and prognostic evaluation in patients with CRC.

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Disclosure

The authors report no conflict of interests in this work.

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