Original Article
The construction of EpCAM/vimentin-PLGA/lipid immunomagnetic microspheres and the isolation of circulating tumor cells from lung cancer

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Abstract: This study aims to investigate the isolation effect of the epithelial cell adhesion molecule (EpCAM) and the vimentin antibody PLGA/lipid magnetic microsphere on the isolation and identification of lung cancer circulating tumor cells (CTC) in the isolation and identification system of lung cancer CTC. The synthesis of the magnetic microsphere was achieved by the composite package of Fe3O4 magnetite nanoparticles with poly (lactic-co-glycolic acid) carboxylic acid endcap (PLGA-COOH) and octadecyl quaternized carboxymethyl chitosan (OQC), immunomagnetic microspheres (IMS) was prepared by linking an EpCAM antibody and a vimentin antibody. Blood samples of tumor-bearing nude mice (A549 lung cancer cells) were collected. Through a separation technique, the CTCs were captured by the EpCAM immunomagnetic microspheres (EpCAM-MS) and vimentin immunomagnetic microspheres (Vim-MS), and the cells were then counted and compared with the pathological condition of the tumor tissues. The results showed that self-prepared EpCAM-MS and Vim-MS could effectively capture lung cancer CTC and match the pathological findings.

Keywords: Immune magnetic microsphere, circulating tumor cell, lung cancer, tumor detection

Introduction
Circulating tumor cells (CTCs) are tumor cells that are spontaneously released from the solid tumor or metastatic foci into the peripheral blood circulation due to diagnostic and surgical procedures and are one of the important causes of postoperative recurrence and distant metastasis in patients with malignant tumors [1-4]. At present, the methods commonly used in the clinical diagnosis and prognosis of tumors depend mainly on the biopsy of tumor tissues, imaging, serum tumor markers, and so on [5-9], which, however, have some limitations in assessing tumor metastasis [2, 10, 11]. At the same time, for the isolation and enrichment of CTCs, only a small amount of peripheral blood needs be taken from patients, without any side effects, which can contribute to the high-frequency monitoring, so as to monitor the progress of the disease in real time. Previous research has shown that in lung adenocarcinoma patients, the CTC count was superior to the serum tumor marker in assessing the efficacy of treatment, and furthermore, CTC is thought to be superior to imaging in the early evaluation of clinical efficacy [12, 13]. Nevertheless, current available and effective measures, as well as the corresponding efficiency of detecting CTC are still insufficient in lung cancer patients [14-16], so further study and development of stable and reliable methods for the detection of CTC is required for lung cancer patients.

The epithelial cell adhesion molecule (EpCAM) shows high expression in most epithelial derived tumor cells, but it lacks expression in blood cells and lymphocytes [1]. At the present time, the differentiation of tumor cells and normal blood cells is preliminarily identified by the expression of EpCAM. However, only 70% of the tumor cells that originate from various tissues express EpCAM, and in most cases, the loss of the epithelial cell specific antigen may result in
a reduction in the efficiency of the single antibody capture of CTCs because of the epithelial mesenchymal transition (EMT) in some tumor cells [10]. Furthermore, vimentin is found to be highly expressed on the surface of EMT transformed tumor cells [17-19], and, in this regard, the vimentin magnetic spheres can be constructed simultaneously to avoid changes in the EMT characteristics of CTC leakage. Poly(lactic-co-glycolic acid) (PLGA) is a biodegradable functional polymer organic compound that is characterized by good biocompatibility, excellent stability, and the ability to be readily ingested by phagocytes [20-24], and it has been approved by the Food and Drug Administration (FDA) for various clinical studies in humans [25]. Unlike the previous polystyrene or lipid materials prepared to serve as magnetic spheres [26], in this study, a typical PLGA/lipid was used as the skeleton material for magnetic spheres to construct immunomagnetic spheres modified by EpCAM and vimentin. The objective of the present study was to explore the sorting ability of the two kinds of immunomagnetic spheres for CTC in lung cancer A549 cells and lung cancer patients, and to evaluate their clinical value preliminarily.

Materials and methods

Materials and instruments

SPF-level Balb/c-nu/nu mice (Shanghai Silaike Experimental Animal Co. Ltd., China); non-small cell lung cancer cell line A549 cells (ATCC cell bank); DMEM culture medium, fetal bovine serum and trypsin (Gibco company, USA); DAPI staining solution (Sigma company, USA); DOPC (Avanti company, Alabaster, USA); CD45-PE (eBioscience company, USA); EpCAM, CK19 and vimentin monoclonal antibodies (Abcam company, Cambridge, UK); magnetite nanoparticle (Ju Kang (Shanghai) biological science and Technology Co. Ltd., China); cholesterol (Chol), dichloromethane and other commonly used reagents (Sinopharm Group Co. Ltd., China); fluorescence microscope (OLYMPUS B×61, Olympus Corp, Japan); flow cytometer (BD FACS caliper, Becton Dickinson, USA).

Methods

Preparation of EpCAM/vimentin-PLGA/lipid immunomagnetic microspheres: Reverse-phase evaporation was used for the preparation of the EpCAM/vimentin-PLGA/lipid immunomagnetic microspheres, in accordance with the specific preparation process and reagent dosage described in previous studies [27-29]. PLGA-NH2, Chol, DOPC and Fe3O4 magnetite nanoparticles were dissolved in chloroform and used as an oil phase; with Tween-80 as surfactant, PBS (pH7.4) at a concentration of 0.1 mol/L was prepared as the aqueous phase. Then in the case of the ultrasonic probe ultrasound, we added the aqueous phase slowly to the oil phase, and the volume ratio of the aqueous phase to the oil phase was 2:1. After a uniform emulsion was formed, we steamed off the excess chloroform on a rotary evaporator, and PLGA/lipid composite magnetic sphere solution was eventually obtained.

Subsequently, the coupling agents EDC and NHS were added to the PLGA/lipid composite magnetic sphere solution, and the mixed solution was then divided into 2 parts after a thorough dissolution, then the EpCAM and the vimentin antibodies were supplemented, respectively. Afterwards, 24 h later, an EpCAM or vimentin modified nano-magnetic sphere was obtained after the magnetic separation, and this is termed EpCAM-PLGA/lipid immunomagnetic microspheres (EpCAM-MS) or vimentin-PLGA/lipid immunomagnetic microspheres (VIM-MS), respectively, both of which were reserved after freezing and drying. See Scheme 1 for the specific preparation process.

The main components of the CTC isolation and identification system for lung cancer included EpCAM/vimentin-PLGA/lipid immunomagnetic microspheres, the FITC labeled CK19 monoclonal antibody (CK19-FITC), a DAPI staining solution, the PE labeled CD45 antibody (CD45-PE), a phosphate buffer solution (PBS, 0.01M, pH = 7.4), and deionized water.

Cell culture and cytotoxicity analysis of vimentin-PLGA/lipid immunomagnetic microspheres

Cell culture: In this study, A549 cells were cultured in a DMEM complete culture medium containing 10% fetal bovine serum (FBS) at 37°C under humid conditions and 5% CO2. The dose distribution in the culture medium was a 3 ml culture medium in a 35 mm culture dish, and 12 ml in a 10 cm culture dish.

Cryopreservation and thawing of cells: The frozen liquid was mixed according to the proportion of fetal bovine serum: DMSO (9:1) and then preserved at 4°C. The cells were digested with
an appropriate amount of 1.25% trypsin, and an appropriate amount of the complete culture medium was added when cells became round but not floated in the culture medium. The cells were dispersed by repeated pipetting to form cell suspension cells, then the cell suspension was centrifuged, and the upper layer culture medium was discarded. Subsequently, the cells were precipitated and stored in a frozen tube after being suspended by cell suspension, and then placed in a gradient cooler, stored overnight in an -80°C ultra-low temperature freezer, followed by re-migration into liquid nitrogen for long-term preservation. For the resuscitation of the frozen cells, the cells were thawed in a water bath at 37°C, and an appropriate amount of complete culture medium was added afterwards. About 5-8 h later, the complete culture medium was replaced, and a conventional passage culture was carried out.

Cytotoxicity analysis of EpCAM and vimentin-PLGA/lipid immunomagnetic microspheres

The digestion of lung cancer cells was achieved with trypsin, and a single cell suspension was prepared and added to a serum containing the medium to neutralize the trypsin. After diluting the cells, the cell concentration was calculated using the cell counting plates. Following cell counting, the lung cancer cells were inoculated into a 96-well plate at a density of 8000 cells/well, and the density of the culture medium was 100 μl per well. After the overnight incubation of the cells, EpCAM-MS or VIM-MS with a concentration-gradient was added separately to the final concentration of 0, 10, 50, 100, 200, 500 and 1000 μg/ml, respectively. The cells were then cultured at 37°C for 0.5 h, 2 h and 24 h, 10 μl 5 mg/mL MTT reagent was then added to each well, and the wells were then placed and incubated in incubator for 3 h, and obvious formazan crystal formation was observed under the microscope. After removing the culture medium, a 150 μl DMSO solution was added to each well to dissolve the crystal. At 490 nm wavelengths, the readings were recorded with an enzyme marker and the statistical results were obtained.

CTC model of phosphate buffer solution (PBS)

A single cell suspension of lung cancer A549 cells was prepared, and after counting, the cell suspension was added to 7.5 ml PBS at five different gradients of 10, 20, 50, 100, and 200 to stimulate the CTCs for the detection of the ability to capture the CTC of prepared EpCAM-MS and VIM-MS. The grouping cell samples of the model under testing were placed in the corresponding centrifuge tubes, and equal amounts of PBS was added for intensive mixing. Subsequently, by adding 20 μl immunomagnetic microspheres, the mixed liquid was then incubated at room temperature for 15 min, with mixing every 5 min. The centrifuge tube was inserted into the magnetic separator to absorb the supernatant for 10 min, and after that, the centrifuge tube was removed. The magnetic separation of the captured CTCs was washed 2 times using PBS. Then, 10 μl of FITC labeled CK19 monoclonal antibody (CK19-FITC), 20 μl of DAPI staining solution, and 10 μl of PE labeled CD45 antibody (CD45-PE) were added and mixed, followed by staining in a dark environment for 15 min. The magnetic separation was performed for 5 min after staining, and the solution was washed two times with deionized water to wash off unbound antibodies and DAPI. Finally, 15 μl of deionized water was added to the centrifuge tube for the re-selection of CTCs, the evenly mixed liquid was then applied to the anti-fall slide glass treated with poly lysine, and when the drip dried, the glass was placed under a fluorescence microscope for cell counting.

Isolation and identification of CTC in the peripheral blood of nude mice bearing lung cancer by EpCAM and vimentin-PLGA/lipid immunomagnetic microspheres

Preparation of the tumor-bearing nude mice model: A subcutaneously transplanted tumor model was established by cell injection, human lung cancer A549 cells were cultured regularly to a certain number, and the cells cultured in vitro in their logarithmic phase were refined by trypsin to form the single cell suspension. Cell counts were made with a cell counting plate, and trypan blue staining was used to detect cell viability, adjusting the cell concentration to 1×10^7/mL when the cell viability reached >95%.

There were a total of 60 nude mice 3-4 weeks old and with a weight of 20 g, half males and half females. The right back and lateral femoral skin of each mouse was disinfected with Anerdian, and then a prepared lung cancer cell suspension at an amount of 0.2 mL (viable count at a density of 1×10^7) was extracted with a 1 mL standard syringe, and injected subcutaneously on the right back skin of each mouse.
Detection of tumor growth index: The size, length, diameter (L) and transverse diameter (W) of the subcutaneous tumor model in the nude mice were measured by Vernier caliper, and the approximate volume of each tumor was calculated according to the formula \( V = L \times W^2 / 2 \). The measurement was performed every 2-3 d to calculate the growth rate. Growth rate = \( \frac{\text{mean tumor volume (mm}^3\text{)/tumor-bearing time of host nude mice (d)}}{} \).

CTC detection of tumor-bearing nude mice: The tumor markers were measured in the successfully established tumor bearing nude mice every 2-3 days. Then, 1 mL blood was taken from an orbit for the detection of CTC.

The collected blood specimens containing anticoagulant were primarily centrifuged at 1000 rpm/min for 10 min. The upper liquid was collected carefully and placed in the EP tube, and then mixed evenly with an equal amount of PBS. An amount of 30 μL lipid immunomagnetic microspheres was added, followed by incubation at room temperature for 30 min, mixing every 10 min. A centrifuge tube was inserted into the magnetic separator, then the centrifuge tube was removed after absorbing the supernatant for 5 min. After washing 3 times with PBS, 30 μL DAPI staining solution, 10 μL CK19-FITC staining solution, and 10 μL CD45-PE staining solution were added and mixed, which were then stained under a dark environment for 15 min. After that, washing 3 times was again conducted with PBS. Finally, 10 μL of deionized water was added to the EP tube for re-suspension, and the mixed liquid was then applied evenly to the anti-fall slide glass treated with poly lysine, and when the drip dried, the glass was placed under a fluorescence microscope for cell counting.

Isolation and identification of CTC in clinical blood samples of lung cancer by vimentin-PLGA/lipid immunomagnetic microspheres

The study was approved by the ethics committee, and different blood sampling plans were identified according to differences in patient diagnosis and treatment after obtaining the informed consent of the patient. Early morning fasting peripheral venous blood samples (7.5 ml) were collected from each outpatient. A total of 15 lung cancer specimens were involved for the isolation and identification of CTC. Anticoagulated blood samples (7.5 mL) collected from the tumor patients were equally divided into three parts, 2.5 mL per part, and then centrifugation at 1000 rpm/min was performed for 10 min. The upper liquid was placed carefully in the EP tube and mixed with the same amount of PBS. Two of them were added with 30 μL EpCAM-MS and 30 μL Vim-MS 30 μL, respectively, and then the remaining blood specimen was separated with 30 μL EpCAM-MS, followed by a secondary separation with 30 μL Vim-MS. Magnetic spheres were incubated at room temperature for 30 min during the process of magnetic sphere separation, with mixing every 10 min. A centrifuge tube was inserted into the magnetic separator for absorbing the supernatant for 5 min. After washing three times with a
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PBS solution, 30 μL DAPI staining solution, 10 μL CK19-FITC staining solution, and 10 μL CD45-PE staining solution were mixed, and the staining was then conducted under a dark environment for 15 min. After that, washing with PBS was conducted three times. Finally, 10 μL deionized water was added to the EP tube for re-suspension, the mixed liquid was then applied evenly to the anti-fall slide glass treated with poly lysine, and when the drip dried, the glass was placed under an OLYMPUS fluorescence microscope for cell counting.

Statistical analysis

SPSS 18.0 statistical software was used to enter and process the data. The inspection level was α = 0.05 (bilateral), and P ≤ 0.05 meant that the difference was significant.

Results and discussion

Preparation and physical characterization of nano-lipid microspheres modified by antibodies

Significantly different from general methods used for the preparation of PLGA nanoparticles, such as the double or singleemulsion method,theresence-phase evaporation method that is commonly used for lipid preparation was involved in the present experiment. Using PLGA and lipid material, the DOPC, as the matrix materials for magnetic spheres, with Tween-80 as surfactant, lipid materials of DOPC and cholesterol were added appropriately to the hard material of PLGA. Meanwhile, oily Fe₃O₄ magnetic nanoparticles were also included in the preparation, so that the prepared

Figure 2. Size distribution of magnetic particles. A. Average size distribution of EpCAM-MS. B. Average size distribution of VIM-MS.

Figure 3. Cytotoxicity of immunospheres on A549 cells. A. EpCAM-MS. B. VIM-MS.
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The UV absorption peaks of the immunomagnetic magnetic spheres prepared in this experiment are shown in Figure 1A, with the characteristic absorption peaks at 280 nm, and the magnetic saturation curve is shown in Figure 1B, and the magnetic saturation values of EpCAM-MS and VIM-MS were both very close to 9.8 emu/g, significantly smaller than that of pure Fe₃O₄ nanoparticle. The total magnetic hysteresis curve of the immunomagnetic sphere was closed and superparamagnetic. The diameter of the nano-magnetic spheres was measured by a particle size analyzer. The average diameter of the magnetic spheres without EpCAM immunization was (187.3±6.3) nm, and the PDI was 0.325 (Figure 2A). Meanwhile, the average diameter of the vimentin immunomagnetic spheres was (192.4±4.1) nm (Figure 2B), and the PDI was 0.213. The smaller particle size, uniform dispersion, and higher magnetic saturation contribute to the stability of the antibody magnetic spheres in aqueous solutions, enhancing the binding efficiency with tumor cells. The antibody magnetic spheres prepared in this experiment met the expected requirements.

Cytotoxicity analysis of vimentin-PLGA/lipid immunomagnetic microspheres

Good biocompatibility and low cytotoxicity are prerequisites for the clinical application of biological nanomaterials. As presented in Figure 3, the constructed EpCAM and vimentin-PLGA/lipid immunomagnetic spheres indicated a relatively low inhibition rate of lung cancer cells at the concentration of 500 μg/mL (<20%), and the cytotoxicity of the lung cancer cell lines increased gradually when the concentration was greater than 1 mg/mL, and the corresponding inhibition rate was about 50%. Therefore, under the normal concentration of microspheres (<500 μg/mL), the constructed PLGA/lipid immunomagnetic spheres showed a low cytotoxicity to the captured cells, laying a foundation for the culture of CTC, the analysis of surface markers, and the cell behavior and gene analyses. Meanwhile, the PLGA applied in the experiment was biodegradable biomaterial, which can be degraded gradually with the re-culture of the cells, and it is eventually metabolized by cells.
Isolation and identification of lung cancer cells in PBS buffer by PLGA/lipid immunomagnetic microspheres

The effects of the PLGA/lipid immunomagnetic spheres on the cells were observed by laser scanning confocal microscopy (Figure 4). We observed that the nuclei of the A549 cells captured by PLGA/lipid immunomagnetic spheres in PBS simulated samples were stained with DAPI and showed a blue fluorescence; meanwhile, a green fluorescence was emitted due to the combination with CK19-FITC. The superimposition of the DAPI and the FITC fluorescence indicated that CK19 was located on the surface of the cells and served as a surface marker for the tumor cells.

As shown in the bright field micrographs in Figure 4, it was obvious that the immunomagnetic spheres interacting with the A549 cells were located on the surface of the cell, and the immunomagnetic spheres were dark brown and adhered uniformly to the extracellular region of the cell. The results documented that the constructed immunomagnetic spheres had a relatively higher ability to bind to the CTC cells, and the immunomagnetic microspheres could actively identify the tumor cells in the aqueous solution. Figure 5 illustrates the recovery efficiency of CTC in simulated samples of different concentration gradients. The results showed that the immunomagnetic spheres had a higher recovery rate for CTC with different concentration gradients.
ranging between 60-91%. The recovery rate of the blood delivery cells was slightly lower than that of PBS, and there was no statistical difference between the groups. Furthermore, there was a better linear relationship between the number of cells recovered and the number of cells released. In the present experiment, the combination of constructed immunomagnetic spheres of EpCAM-MS and VIM-MS could increase the recovery efficiency of the cells significantly (P<0.05). Importantly, the immunomagnetic spheres constructed in the experiment lay the foundation for further clinical applications.

Isolation and identification of CTC in the peripheral blood of nude mice bearing lung cancer by vimentin-PLGA/lipid immunomagnetic microspheres

Tumor growth volume parameters and the corresponding CTC numbers were observed and recorded in the established tumor model for 8 weeks. The experimental results are shown in Figure 6. After two weeks of implantation, there was an increase in CTC, and captured CTC increased gradually as the tumor grew, which reached more than 8 in the eighth week. Correspondingly, the weight of the nude mice increased in the first 3 weeks (Figure 6B), and later, as time went on, the weight did not increase but showed a decreasing trend (Figure 6B). The data related to the tumor volumes are shown in Figure 6C, and there was a congruent relationship between tumor volume and the number of CTCs. This experiment provides a potential reference for the further development of clinical trials.

Isolation and identification of CTC in clinical blood samples of lung cancer using two kinds of immunomagnetic microspheres

Clinical results regarding the immunomagnetic spheres for the clinical samples of lung cancer patients are shown in Figure 7A. The corresponding results observed under fluorescence microscopy showed that the CTC positive lung cancer cells were bright green in color from the fluorescence caused by the CK19-FITC antibodies, while the DAPI staining had a blue fluorescence, and the CD45 staining did not show any fluorescence. Meanwhile, the surfaces of the cells were covered by a dark brown nano-magnetic sphere, fully or partially, which might be positive lung cancer CTC. The CTC numbers of

Scheme 1. Schematic of the preparation of EpCAM-MS or VIM-MS.
the 15 patients with lung cancer were statistically analyzed, and the results revealed that the positive rate of combined application of the two kinds of magnetic spheres was 95%, significantly higher than that of the single use of the immunomagnetic sphere, which was 68% (EpCAM-MS group) and 73% (Vim-MS group), respectively (Figure 7B). The above results suggest that the simultaneous application of EpCAM and two kinds of magnetic spheres targeting EMT conversion can effectively improve the sorting efficiency of CTC and reduce the leakage rate. The number of CTC cells sorted out by each magnetic sphere was also strongly correlated with the clinical stage and the degree of metastasis of the tumor, which remains to be further explored by expanding the sample quantity in a later study.

Conclusion

In this study, we demonstrated how EpCAM/Vimentin-PLGA/lipid immunomagnetic spheres are prepared by employing PLGA/lipid to coat magnetic spheres, followed by the modification of anti-EpCAM or vimentin antibodies on the shell of the PLGA/lipid. The physical characterization of the magnetic spheres showed that they have stronger magnetism and smaller particle size. Furthermore, the prepared immunomagnetic spheres have a relatively higher CTC separation ability, which is proved by the recovery experiments of the A549 cell lines in a simulated PBS solution. Simultaneously, the results of the CTC separation in the blood of lung cancer patients demonstrate that the staining effect is clear and distinguishable. Meanwhile, compared with the clinical examination results of the hospital, the two kinds of immunomagnetic spheres constructed in this study have a relatively high CTC detection rate and a low false positive rate, suggesting that the combination of the two kinds of immunomagnetic spheres had a higher detection efficiency for CTC in the blood of patients with lung cancer, providing a certain reference significance for clinical diagnosis and condition monitoring.

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Disclosure of conflict of interest

None.

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