Abstract. Esophageal cancer is a malignant tumor with a relatively high invasiveness, metastatic potential and worldwide incidence among human cancers. The majority of patients with esophageal cancer are diagnosed in a late tumor stage due to a lack of advanced and sensitive protocols for the diagnosis of patients with early-stage esophageal cancer. In the current study, contrast-enhanced computerized tomography (CECT) combined with Chitosan-Fe$_3$O$_4$ nanoparticles targeting fibroblast growth factor receptor (FGFR) and vascular endothelial growth factor receptor (VEGFR; CECT-CNFW) were used to diagnose patients with suspected esophageal cancer. A Chitosan-Fe$_3$O$_4$-parceled bispecific antibody targeting FGFR and VEGFR was produced and its affinity to esophageal cancer cells was determined both in vitro and in vivo. A total of 320 patients with suspected esophageal cancer were voluntarily recruited to evaluate the efficacy of CECT-CNFW in the diagnosis of early-stage esophageal cancer. All participants were subjected to CT and CECT-CNFW to detect whether tumors were present in the esophageal area. A Chitosan-Fe$_3$O$_4$ nanoparticles contrast agent was orally administered at 20 min prior to CT and CECT-CNFW. The results demonstrated that CECT-CNFW improved diagnostic sensitivity and provided a novel protocol for the diagnosis of tumors in patients with suspected esophageal cancer at an early-stage. Furthermore, the resolution ratio of images was enhanced by CECT-CNFW, which enabled the visualization of tiny tumor nodules in esophageal tissue. Clinical data demonstrated that CECT-CNFW diagnosed 200 patients with suspected early-stage esophageal cancer and 120 patients as tumor-free. In addition, CECT-CNFW exhibited higher signal enhancement of tumor nodules than CT, suggesting a higher accuracy and accumulation of nanoparticle contrast agent within the tumor nodules of esophageal tissue. Notably, the survival rate of patients with esophageal cancer diagnosed at an early-stage by CECT-CNFW was higher than the mean five-year survival rate (P<0.01). In conclusion, CECT-CNFW enhanced the sensitivity and accuracy of CT in the diagnosis of early-stage esophageal cancer. Thus, CECT-CNFW may improve the accuracy of CT in the diagnosis of mural enhancement in patients with esophageal cancer.

Introduction

Esophageal cancer is the most common malignant tumor of the digestive tract (1). Clinical data has demonstrated that, worldwide, ~300,000 cases of esophageal cancer result in mortality each year (2,3). A previous systematic review and meta-analysis evaluated the health-related quality of life of patients with esophageal cancer following potentially curative treatment and results identified that health-related quality of life of patients with esophageal cancer is an indicator to evaluate the efficacy of anti-cancer drugs (4). Although increasing cancer therapy technologies continue to be investigated in clinical studies, the five-year survival rate of patients with esophageal cancer is lower than 12.5% (5). Previous reports have indicated that C-X-C motif chemokines, their cognate receptors and tumor metastasis-associated proteins are involved in the pathogenesis of human esophageal cancer (6,7). In addition, lymph node, lung and stomach metastases are key prognostic indicators in late-stage esophageal cancer, with metastatic tumor cell expansion leading to a shortened survival rate (8-10). Therefore, the prevention and early diagnosis of esophageal cancer may enhance the cure rate and improve survival in patients with early-stage esophageal cancer.

Currently, the majority of patients clinically diagnosed with esophageal cancer are in an advanced stage of the disease (11). Although a number of reports have introduced various diagnostic techniques for early-stage esophageal
cancer, including Raman spectroscopy, chemometric techniques, image-enhanced magnifying endoscopy, endoscopic ultrasound elastography and contrast-enhanced computerized tomography (CECT). CECT is the most frequently used method for the diagnosis of esophageal cancer and lymph node metastasis (12-14). Although CECT is considered to have many advantages, its relatively low resolution makes it inconclusive in the final diagnosis of patients with suspected cancer (15,16). Due to the lack of data on early-stage cancer, comprehensive treatments and effective therapeutic plans have not been developed for patients with suspected early-stage cancer (17,18).

Clinically, contrast agent improves the resolution of CT, and the most commonly used contrast agents are employed during ultrasound examination, due to their resonance following exposure to ultrasound waves (19,20). Previous results have indicated that contrast agent combined with CT may be used for the diagnosis of tumor stage and progression (21). More recently, nanoscale microbubbles of contrast agents have been developed and clinically applied in disease diagnosis (22). Therefore, nanoscale microbubbles specific to the tumor markers of esophageal cancer may improve the diagnostic sensitivity and resolution of CT in the diagnosis of patients with early-stage or suspected esophageal cancer.

Previous reports have suggested that fibroblast growth factor receptor (FGFR) is overexpressed in tumor cells, and tumor vasculature may be regulated by FGF/FGFR signaling-mediated angiogenesis and bone marrow-derived cell recruitment (23,24). Previous results have also indicated that vascular endothelial growth factor receptor (VEGFR) is overexpressed and associated with tumor growth, aggressiveness and tumor angiogenesis in the progression of human cancers (25,26). In the present study, the efficacy of CECT combined with nanoscale microbubble contrast agent targeting of FGFR and VEGFR was investigated in patients with suspected esophageal cancer. Results of this clinical analysis demonstrated the potential applications of CECT combined with Chitosan-Fe$_3$O$_4$ nanoparticles targeting FGFR and VEGFR (CECT-CNJV) for improving imaging modality and diagnostic sensitivity in the diagnosis of esophageal cancer. The present outcomes indicated that CECT-CNJV improved image resolution and diagnostic accuracy for the early diagnosis and final confirmation of suspected cases, suggesting that CECT-CNJV may be a potential diagnostic method for early-stage esophageal cancer.

**Materials and methods**

**Ethics statement.** The present clinical design (approval number: DPHSP20080124M) was carried out in accordance with the recommendations in the Guide for the Care and Use of Clinical Study of the Pharmaceutical Administration Law of China (27). The present study was approved by the Ethics Committee of Dongying People's Hospital (Dongying, China). All clinical examination and testing procedures were performed according to standard operating procedures, and all patients provided written informed consent.

**Patients and volunteers.** A total of 320 patients with suspected esophageal cancer aged 24.3-75.4 years old from Dongying People's Hospital (Dongying, China) were enrolled in the present study between May 2014 and January 2016. Among these patients, 156 were male and 164 were female. Control tissues and cells were obtained from 6 patients (3 male and 3 female) who were diagnosed as tumor free. The mean age of control patients was 42.5 years old. All patients were subjected to CECT and CECT-CNJV for the detection of early-stage esophageal cancer. The characteristics of patients with suspected esophageal cancer are summarized in Table I. After diagnosis by CECT and CECT-CNJV, patient survival was investigated in a 60-month long-term observation period. All patients were investigated and patients were assessed every two months.

**Nanoparticles contrast agent.** Novel Chitosan-Fe$_3$O$_4$ nanoparticles encapsulated by bispecific antibody against FGFR and VEGFR (BisFV) were used as a CNFV contrast agent to improve the imaging resolution ratio and diagnostic sensitivity of early-stage esophageal cancer diagnosis. BisFV antibody was a gift from Professor Yuan Hui of the Biological Pharmaceutical Laboratory at Shandong University of Science and Technology (Qingdao, China) Chitosan-Fe$_3$O$_4$-encapsulated BisFV was manufactured according to the covalent bonding method described in a previous study (28). The CNFV contrast agent (1-10 mg/kg, n=10; 10-25 mg/kg, n=14; 25-40 mg/kg, n=18) was orally taken at 20 min prior to CECT-CNJV to allow adherence to esophageal cancer cells. Following the 20 min pretreatment, CNFV was visualized using a CECT system. The signal intensity was analyzed using CECT system.

**Pharmacodynamics of BisFV.** Plasma concentration of BisFV was analyzed in patients with suspected esophageal cancer following treatment with CNFV contrast agent. Blood samples were collected from 32 participants at 0, 6, 12, 18 and 24 h following administration of CNFV contrast agent. Plasma BisFV levels were determined using liquid chromatography-tandem mass spectrometry as described previously (29).

**Scan protocol.** A 64-multidetector CECT diagnosis system (Philips Medical Systems, Inc., Bothell, WA, USA) was used to measure the efficacy of CNFV using a preprogrammed setting. The preprogrammed setting was optimized to record the best image formation. The esophagus of all patients was scanned by CECT, according to the instructions of the CECT system. The parameters and settings of CECT were as described in a previous study (30). CECT and CECT-CNJV imaging was performed in all patients with suspected esophageal cancer.

**Image data analysis.** Data from the CECT-CNJV and CECT image sets were analyzed using a CT system (Version 2.3, Shimadzu, Kyoto, Japan). Esophageal cancer masses were detected in the CECT or CECT-CNJV images. The patients with suspected early-stage gastric cancer were analyzed CECT-CNJV. The sizes of tumor nodules were evaluated in regions of stomach tumor lesions using the CT system.

**Treatment of esophageal cancer patients diagnosed by CECT-CNJV.** Following diagnosis of early-stage esophageal cancer using CECT-CNJV, patients immediately received different treatments following diagnosis, including...
radiotherapy, chemotherapy, Chinese medicine, biological therapy and comprehensive therapy (Table II). The median overall survival and median progression-free survival of patients with esophageal cancer were analyzed according to a previously described method (31).

**Western blot analysis.** Western blot analysis was performed as previously described (32). Tumor cells were lysed in lysis buffer containing protease-inhibitor (cat no. P3480, Sigma-Aldrich; Merck KGaA) and were centrifuged at 8,000 g at 4°C for 10 min. Protein concentration was measured using a BCA protein assay kit. Protein samples (20 µg) were resolved using 15% SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Merck KGaA). After 1 h blocking at 37°C using 10% blocking reagent (Roche Applied Science, Penzberg, Germany), membranes were incubated with primary antibodies: mouse anti-human FGFR (1:1,000, cat no. ab10646), mouse anti-human VEGFR (1:1,000, cat no. ab2349) and β-actin (1:1,000, cat no. ab124721, all Abcam, Cambridge, UK) for 12 h at 4°C. Membranes were then washed three times in TBST and incubated with HRP-conjugated Immunoglobulin G mAb (1:2,000; cat no. PV-6001; ZSGB-BIO, Beijing, China) for 2 h at 37°C. Following three washed in TBST, membranes were developed using a chemiluminescence assay system (Roche) and exposed on Kodak exposure films. Densitometric quantification of the immunoblot data was performed using the software of Quantity-One (Bio-Rad Laboratories, Hercules, CA, USA).

**Immunofluorescence staining.** After esophageal cancer was confirmed in patients by CECT-CNFP, esophageal tumor cells isolated from patients on day 7 using tumor resection were cultured in minimum essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in vitro. Esophageal tumor cells were incubated with mouse anti-human FGFR (1:1,000, cat no. ab10646, Abcam, Cambridge UK) or mouse anti-human VEGFR (1:1,000, cat no. ab2349, Abcam) for 2 h at 37°C, then washed three times with phosphate-buffered saline. Cells were incubated with red fluorescence-labeled goat anti-mouse (1:1,000, cat no. ab150117, Abcam) or red fluorescence-labeled (1:1,000, cat no. ab150115, Abcam) goat anti-mouse IgG for 30 min at 37°C. Esophageal tumor cells were observed under a fluorescence microscope. The immunofluorescence procedures have previously been reported in detail (33).

**Immunohistochemistry.** Esophageal tumors and normal esophageal tissues (tumor free individuals) from patients were fixed with 10% formaldehyde for 2 h at 37°C, then embedded in paraffin and cut into tumor 4 µm thick sections. Antigen retrieval was performed on the tumor sections using eBioscience™ IHC Antigen Retrieval Solution (cat no. 00-4955-58, Invitrogen; Thermo Fisher Scientific, Inc.), sections were washed with PBST (Sigma-Aldrich; Merck KGaA) and subsequently incubated with mouse anti-human FGFR (1:1,000, cat no. ab10646, Abcam) or mouse anti-human VEGFR antibodies (1:1,000, cat no. ab2349, Abcam) for 12 h at 4°C. Following antibody incubation, proteins were washed with PBST three times and incubated with Alexa Fluor 488-labeled secondary antibodies (1:500; Beyotime Institute of Biotechnology, Haimen, China) for 2 h at 37°C and the

### Table I. Characteristics of patients with esophageal cancer.

| Characteristic         | Male       | Female      |
|------------------------|------------|-------------|
| Number, n              | 156        | 164         |
| Age, range             | 24.3-66.6  | 26.3-75.4   |
| Medical history of cancer | 2          | 3           |
| Blood pressure (mm Hg) | 108.4±17.2 | 103.5±16.1  |
| Blood glucose (mmol/l) | 7.2±3.7    | 8.2±3.2     |

### Table II. Treatment of patients with esophageal cancer diagnosed by contrast-enhanced computed tomography combined with Chitosan-Fe₃O₄ nanoparticles targeting fibroblast growth factor receptor and vascular endothelial growth factor receptor.

| Variable               | Male    | Female   |
|------------------------|---------|----------|
| Number, n              | 56      | 64       |
| Treatment              |         |          |
| Radiotherapy           | 12      | 16       |
| Chemotherapy           | 14      | 12       |
| Chinese medicine       | 15      | 18       |
| Biological therapy     | 15      | 18       |
| Comprehensive therapy  | 34      | 45       |

### Table III. Confirmation of contrast agent nanoparticle dosage for patients with esophageal cancer.

| Variable | Dosage, mg/kg |
|----------|--------------|
|          | (n=10)       | (n=14) | (n=18) |
| Signal intensity (HU) | 56.2±8.8 | 86.7±10.3 | 83.5±12.5 |
| Sensitivity (%)        | 65.6±12.2 | 81.2±12.6 | 82.6±11.6 |
specimens were visualized. A Diaminobenzidine staining system (D7679MSDS, Sigma-Aldrich; Merck KGaA) was used to detect target protein expression according to manufacturer's protocol. For histological staining, tumor sections were stained with hematoxylin and eosin and observed using a light microscope (Olympus BX51, Olympus Corporation, Tokyo, Japan) as described previously (34).

Statistical analysis. Data were presented as the mean ± standard deviation of triplicate experiments. All data were analyzed using SPSS Statistics 19.0 (IBM Corp., Armonk, NY, USA). Unpaired data were analyzed using a Student's t test and comparisons between the data of multiple groups were performed using one-way analysis of variance followed by Dunnett's t test. P<0.05 was considered to indicate a statistically significant difference. Kaplan-Meier analysis was used to estimate the survival rate of patients from a 60-month long-term observation.

Results

FGFR and VEGFR expression in the tumor cells of esophageal cancer patients. The expressions of FGFR and VEGFR in the tumor cells of patients with esophageal cancer were assessed. As depicted in Figs. 1 and 2, the expression and plasma levels of FGFR and VEGFR were upregulated in the tumor cells of esophageal cancer patients when compared with normal esophageal cells (P<0.01). Immunohistochemistry also indicated that FGFR and VEGFR were upregulated in clinical esophageal tumor tissues when compared with normal esophageal tissues (Fig. 3). Furthermore, immunofluorescence demonstrated that FGFR and VEGFR were expressed on the surface of esophageal tumor cells (Fig. 4). These results suggest that FGFR and VEGFR are upregulated in the tumor cells and tissues of patients with esophageal cancer.

Efficacy of CECT-CNLFV in the early diagnosis of patients with suspected esophageal cancer. The affinity of BisFV for FGFR and VEGFR was evaluated. As depicted in Figs. 5 and 6, Chitosan-Fe$_3$O$_4$-encapsulated BisFV was able to bind FGFR and VEGFR, as determined by ELISA. The dose of nanoparticles that achieved optimum targeting efficiency was identified as 25 mg/kg (Table II). Subsequent analysis of patient clinical outcomes showed that 120 patients (37.50%) were diagnosed as tumor-free and 200 patients (62.50%) were confirmed to have esophageal cancer after diagnosis with CECT-CNLFV. By contrast, the CECT method
only diagnosed 45 patients (14.06%) with esophageal cancer (Fig. 7). These outcomes demonstrated that CECT-CN.FV
presented significantly higher diagnostic efficacy compared with CECT (P<0.01).

**Pharmacodynamics of CNFV in the plasma of patients with suspected esophageal cancer.** The pharmacodynamics of BisFV was assessed in the plasma of patients with suspected esophageal cancer. Results indicated that the plasma concentration of BisFV was increased and metabolized within 24 h using liquid chromatography-tandem mass spectrometry (Fig. 8). Patients that underwent CECT-CNFK exhibited reduced plasma concentrations of FGFR and VRGFR, both of which reached a minimum at 16 h post-treatment compared with the plasma concentrations prior to diagnosis (Fig. 9).

**Accuracy of CECT-CNFK in the diagnosis of esophageal cancer.** Histopathological analysis was performed to confirm that CECT-CNFK had successfully diagnosed esophageal cancer. The different types of early-stage esophageal cancer in patients, namely medullary, fungating, ulcerative and constrictive, were identified by immunohistochemistry (Fig. 10). A total of 120 cases of esophageal cancer were identified by immunohistochemistry, and the incidence rates of medullary, fungating, ulcerative and constrictive type esophageal cancer were 26.67% (32 cases), 20.00% (24 cases), 25.00% (30 cases) and 28.33% (34 cases), respectively (Fig. 11). As CECT-CNFK also identified 120 cases of esophageal cancer, these data suggest that CECT-CNFK may be a useful clinical method for the diagnosis of early-stage esophageal cancer.
Survival rate of patients with esophageal cancer. Patients diagnosed with early-stage esophageal cancer received different treatments to inhibit or eradicate tumor growth (Table III). The overall survival rate of patients with esophageal cancer following diagnosis with CECT-CNFW was subsequently evaluated. At a 60-month follow-up, 92 patients (76.67%) had survived and were tumor-free, and 20 patients (16.66%) had survived with tumors. A total of 8 patients (6.67%) did not survive (Fig. 12). The median overall survival rate was 55.2 months (Fig. 13) and median progression-free survival rate was 44.6 months (Fig. 14). These data suggest that patients with early-stage esophageal cancer diagnosed by CECT-CNFW and administered with anti-cancer treatments present with longer survival rates.

Discussion

Early diagnosis of cancer is a significant challenge in the clinical treatment of human cancer (35,36). In recent years, contrast-enhanced ultrasound, CT and fluorodeoxyglucose–positron emission tomography have been widely used in the diagnosis of human cancers (37). In particular, CECT is the most widely used method in the diagnosis of humans tumors (38,39). However, the accuracy and sensitivity of CECT is insufficient in the clinical detection of early-stage tumors (40,41). In the present study, a target nano-scale contrast agent combined with CECT was used to improve the accuracy and sensitivity of CECT in the diagnosis of patients with suspected early-stage esophageal cancer. The target nano-scale contrast agent was Chitosan-FeO$_3$ nanoparticles encapsulated by BisFV, which may bind to esophageal cancer cells. The results indicated that CECT-CNFW not only improved the resolution ratio of images captured by CECT, but also increased the accuracy and sensitivity of CECT in the diagnosis of patients with suspected early-stage esophageal cancer.

Theoretically, a nano-scale contrast agent may improve in vivo tumor imaging made by ultrasound, CT and magnetic resonance imaging (42-44). Kim et al (42) demonstrated that ultrasound enhanced-contrast agents, which may go preferentially to the target tumor tissue and amplify the ultrasound imaging signal in vivo, improved the detection limit of ultrasound imaging. Furthermore, Ding et al (43) demonstrated that targeted Fe-filled carbon nanotube as a multifunctional contrast agent improved the resolution ratio of magnetic resonance imaging of tumors in living mice. These reports indicate that nano-scale contrast agents may be useful for detecting tumor masses in early-stage cancer.

In the present study, a novel nano-scale contrast agent composed of Chitosan-FeO$_3$ nanoparticles encapsulated by BisFV was introduced to evaluate the efficacy of CECT-CNFW in the diagnosis of patients with suspected esophageal cancer. Barium sulfate and iodinated contrast media are frequently used for angiography studies and in the detection of tumors in the digestive system (45,46). Various kinds of electro-positive iron and iron oxide nanoparticles have been used as contrast media in combination with ultrasound, CT and magnetic resonance imaging for the clinical diagnosis of human cancers (47,48). In addition, targeted contrast agents are considered to enhance optical coherence tomography and improve the accuracy of CT in the diagnosis of tumor tissue masses (49). However, although contrast media improve the accuracy of CT to a certain degree, their sensitivity has not been improved in previous studies (21,50). The present results indicated that CECT-CNFW was efficient in the targeting of FGFR and VEGFR, and improved the accuracy and sensitivity of CT in the diagnosis of early-stage esophageal cancer. Notably, long-term follow-up investigations suggested that patients diagnosed by CECT-CNFW at early-stage presented with higher median overall survival and median progression-free survival rates compared with the mean survival rate of patients with esophageal cancer in previous reports (51).

In conclusion, the present study investigated the efficacy of CECT-CNFW in the diagnosis of suspected early-stage esophageal cancer. Chitosan-FeO$_3$ nanoparticles encapsulated by BisFV not only improved the image resolution generated by CT, but also enhanced the accuracy and sensitivity of CT in the diagnosis of early-stage esophageal cancer. These outcomes indicate that CECT-CNFW may be an efficient clinical method for diagnosing patients with suspected esophageal cancer at an early-stage. Thus, CECT-CNFW may be a reliable and sensitive assessment method in the clinical diagnosis of cancer patients.

Competing interests

The authors declare that they have no competing interests.

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