Abstract. Detection of gonadotropin-releasing hormone (GnRH) also known as luteinizing hormone-releasing hormone (LHRH) in the relevant tumor tissue and normal tissues and organs in vivo expression was investigated. To examine the method of direct radio labeling of LHRH by $^{99m}$Tc with relatively high radiochemical purity and stability, screening the best labeling conditions, to establish a simple and reliable method of preparation of $^{99m}$Tc-LHRH was undertaken. The detection of radioisotope-labeled LHRH distribution in mice, LHRH receptor imaging for the study and treatment of cancer basis were evaluated. i) Immunohistochemical staining test was used in 23 patients with hepatocellular carcinoma (HCC), 20 patients with breast cancer, 10 patients with prostate cancer, 20 patients with lung cancer, 20 patients with endometrial cancer tumor cells and normal tissue LHRH-R De Biao da levels; ii) pre-tin method use direct labeling of LHRH, marking completion of saline or human serum were added at room temperature, the chromatography was measured at different times, to calculate the rate of labeled product and the radiochemical purity of the label, in vivo observation of its stability, and comparative analysis of selected optimal condition; iii) rat pituitary cell membrane protein, the product of in vitro radio-receptor marker analysis, through the saturation and inhibition experiments, was used to test its receptor binding activity; iv) Ch-T method labeled $^{125I}$-LHRH, tail vein injection of normal mice at different times were sacrificed, blood and major organs were determined and calculated per gram organization percentage injected dose rate (%. ID/g). Detected by immunohistochemistry in 23 cases of HCC in the LHRH-positive rate was 82.61%, in the corresponding normal tissues, the positive rate was 15.79%; 20 cases of endometrial cancer positive rate of 80% in the corresponding normal tissues was 16.67% positive. $^{99m}$Tc-LHRH mark was 97.9-100.0%, the radiochemical purity of 93.9-96.4%, marking the reaction gel content of <5%. Great product receptor marker analysis showed $^{99m}$Tc-LHRH with saturable receptor binding characteristics and inhibition, and high affinity, $RT = 23.2174 \text{ pmol}$, $KD = 0.4348 \text{ mmol}$; intravenous injection of $^{131I}$-LHRH within 72 h after the mice rapidly cleared the blood radioactivity, the major radioactivity accumulation in the liver and kidneys and by the liver, renal clearance, and other tissues and organs of the radioactivity gradually decreased with time. In conclusion, i) the liver, lung, breast, prostate, endometrial cancer exist in both LHRHR; ii) $^{99m}$Tc-LHRH preparation is simple, rapid, radiochemical purity product obtained higher marks, better stability, no further purification; and iii) LHRH $^{99m}$Tc labeled, still has a high receptor binding ability, biological activity; and has an ideal and realistic dynamics in animals, there is hope, as with the clinical value of imaging agent of GnRH receptors.

Introduction

Worldwide, the incidence and mortality rate of cancer are on the increase, with an increasing number of young cancer patients. Early detection, early diagnosis and early treatment is the key to reducing the mortality rate. Application of many new technologies significantly increases the accuracy of early detection and diagnosis of lesions (1). Currently, exploration of the cause, development mechanisms and laws of the tumor has been elevated to the molecular and genetic level. Oncology imaging study has developed from simple anatomic description to functional imaging, molecular biology and pathophysiological development, and combined with biological markers or gene technology, can detect and determine the characteristics of the disease at the cell and molecular level (2).

Luteinizing hormone-releasing hormone (LHRH) is a decapptide hormone, which is secreted by the hypothalamus arcuate nucleus neurons and transported by axoplasm flow of nodule-pituitary axis to nerve endings at the uplift, and then by hypophysyal portal veins, it flows into the anterior pituitary gland with blood to stimulate the pituitary cells to secrete luteinizing hormone and follicle-stimulating hormone for regulating anterior pituitary gonadotropin secretion, which in turn stimulates the secretion of luteinizing hormone and follicle-stimulating hormone to regulate the level of gender hormones, in order to act on the reproductive axis and regulate the formation of gametes and gonadal endocrine (3,4). It must
be combined with high affinity transmembrane receptors to play its role, and these receptors belong to seven transmembrane receptor families (5-8). LHRH and its receptor are also found in the central nervous system (CNS) and peripheral tissues, indicating that in addition to the release of gonadotropin, this kind of decapptide hormone also has other functions. It has been previously reported that LHRH is generated outside the hypothalamic-pituitary axis and plays specific biological effects outside the hypothalamic-pituitary axis (9). Previous findings have shown LHRH binding sites on tumor cells of ovary, breast, and endometrium, prostate and pituitary, and a high expression of LHRH-R on the tumor cells of the pancreas, lung and liver (10-17). Expression of LHRH-R in the corresponding normal tissue is relatively low. Emmons et al first proved that approximately 80% of ovarian cancer tissues possess binding sites of specificity LHRH, LHRH agonist or antagonist analogues, which indicated the existence of the high affinity LHRH receptor in human ovarian cancer (7). Some LHRH agonists have been used in the treatment of ovarian cancer. Eidne et al first proved the existence of LHRH binding sites on human breast cancer cells (18). Subsequent literature reported the MTX mouse breast cancer cell nuclei has LHRH-R distribution (19). Human breast cancer cell lines MDA2MB231 and ZR27521 cell extracts cultured in vitro showed LHRH immunoreactive (20). Rusiecki et al observed LHRH receptor expression in breast cancer and tissues adjacent to cancer and the relationship between LHRH receptors, estrogen (ER), and progesterone receptor (PgR). The study selected pathological tissue of 90 cases of breast cancer patients, and randomly selected 40 cases of normal tissue adjacent to normal tissue (45 vs. 39%), the results showed that LHRH-R expression is related to tumor tissue with positive PgR (21). In addition, LHRH-R expression of premenopausal breast cancer patients without menstruation is higher (56 vs. 32%, the latter is the value of normal humans) (22). Although LHRH analogues for the treatment of endometrial cancer has a long history, the study on LHRH and its receptor expression in endometrial cancer tissue has just started in recent years, and study of its therapeutic mechanism has been a hotspot over the past 2-3 years. Imai et al found that LHRH stimulation can induce apoptosis in some human reproductive tract tumor cells mediated by Fas ligand. The incidence of primary liver cancer has a significant gender difference (male:female = 9:1 to 7:1), and experimental study and clinical observations have suggested that liver cancer may be a hormone-dependent tumor (23). Recently, LHRH-R gene in human hepatocellular carcinoma (HCC) has been successfully cloned, with investigators confirming that the complementary DNA libraries of LHRH and its receptor produced by PCR reaction and human placenta and pituitary gland LHRH receptor are identical through DNA blot analysis with endogenous oligonucleotide primers as a probe (24). This shows that, LHRH and LHRH-R non-germline mRNAs are widely distributed in normal or malignant tissues that do not belong to reproductive system (25,26). The adjacent piece immunohistochemical double labeling method also shows LHRH in HCC may regulate the growth and differentiation of liver cancer cells in autocrine manner (OS). Katsuno et al found the expression of LHRH on hypophysis growth hormone cell tumors by means of immunohistochemistry and in situ hybridization (27). Some researchers have applied LHRH to the clinical treatment of pituitary adenomas, and found that after a long-term treatment (6 months), pituitary tumor was significantly reduced. This suggests that LHRH can inhibit the growth of pituitary tumor cells (28). The above facts show that normal human pituitary and pituitary tumor cells can express LHRH and its receptor, and that LHRH in pituitary tumor cells may act as an autocrine or paracrine regulator to fulfill its function (28,29). Among tumors derived from non-gonadal axis, pancreatic cancer is a malignant tumor relatively closely connected to LHRH. It has been demonstrated that there are LHRH-R distribution on hamster pancreatic cancer tissue induced by N-dinitroso dioxopropyl two amines (BOP) and human pancreatic tissue through autopsy; and no LHRH binding sites was detected on normal hamster and human pancreatic cancer tissue (30). Szepeshazi et al (31) applied bombesin gastrin-releasing peptide (GRP) agonist RC23095, growth hormone releasing somatostatin analogues RC2160 or LHRH agonists cetrorelix to hamsters induced by nitrite, 8 weeks later, they discovered that epidermal growth factor (EGF) expression in carcinoma tissue decreased by 71 and 69%, respectively. This indicates that EGF may be involved in the adjustment of inhibitory effect LHRH exerts on pancreatic tumor cell growth (32,33).

The identification of LHRH analogs (LHRH-a), activator (LHRHag), and antagonist (LHRHanta) has promoted the study on physiological function of LHRH and its receptor. LHRH is a neurotransmitter in the CNS and the sympathetic nervous system; a paracrine regulator in the gonads and placenta; an autocrine regulator in some tumor cells (19,34,35). Human LHRH-R is composed of 328 amino acids and 7 transmembrane domains, which belongs to G protein-coupled receptors. Junction station of LHRH-R and LHRH in mammalian pituitary gonadal cells has high specificity (36). Photoaffinity labeling, and chromatographical electrophoresis have proved that LHRH-R is a glycoprotein of M-6000, and has 3 N-glycosylation sites, the possible interaction between acidic amino acids at 90, 98 and 29 and arginine in LHRH enables LHRH to play its physiologic adjustment function (37). LHRH-R can also be generated and expressed in hypothalamic-pituitary axis, for example, LHRH-R mRNA expression can be found on human ovarian granulosa, luteal cells, testicular interstitial cells, breast tissue, prostate and rat gastrointestinal epithelium and glandular epithelial cells, suggesting that these LHRH target organs can synthesize LHRH-R by themselves. The presence of LHRH or analogue activates LHRH-R to perform different functions by G protein-coupled phosphoinositide pathway and mediated calcium mobilization, for instance, LHRH in the digestive tract and circular blood may be a sort of gastrointestinal hormone, which can not only play an indirect regulation through the vagus nerve on functions of gastrointestinal system, but also plays a direct role in the regulation of the digestive system through the binding with LHRH-R on mucosal epithelium and epithelial cells (38-40).

Studies over the past decade also found that, LHRH and its receptors are involved in the occurrence and development of some tumors, especially certain tumors with the non-gonadal axis organ origin. For example, LHRH-R also exist in lung cancer, kidney cancer and liver cancer cells. Previous findings showed that LHRH-R expression in gland cancer cells may
be a common phenomenon (41). Additionally, it was identified that, LHRH and its receptor inhibits proliferation of cancer cells, whose expression is closely related to differentiation degree of cancer tissue, i.e., the higher the differentiation degree, the higher the expression of LHRH and its receptors, whereas, the poorer the differentiation degree, the lower the LHRH and its receptor expression (42).

There is evidence showing that LHRH can directly inhibit the proliferation of certain hormone-sensitive tumors, and although it is known that such physiological stimuli leading to cell death is firstly achieved by apoptosis, its antitumor molecular mechanism remains unclear. Some scholars believe, LHRH inhibitory effect on tumor is realized by a mechanism independent of the release of pituitary gonadotropin. Nevertheless, antitumor effect and its targeted therapeutic value of LHRH and its analogues have been widely demonstrated in clinical applications. Studies have reported that the treatment of malignant tumors with high expression of corresponding receptors by combining LHRH targeting with biological toxins or nanoparticle technology has been used in clinical application (32,33).

High expression of a variety of aforementioned pathological conditions provides a basis for using LHRH receptor for imaging. However, studies on LHRH receptor imaging applied to cancer diagnosis are scarce. There is some literature on the characteristics of labeling method and labeled product of radionuclide-labeled LHRH of $^{68}$Ga, $^{123}$I, $^{18}$F, $^{99m}$Tc, some labeled products have entered the pharmacological evaluation stage; there is other literature examining the possibility of the corresponding labeled product serving as developing agent in SPECT, PET imaging, some findings suggested that LHRH would have a broad prospecting receptor imaging of malignant tumors with high corresponding receptor expression (34). However, most such studies still remain at the stage of labeling conditions and involve no further study of malignant tumor cells with high corresponding receptor expression.

Application basis of LHRH receptor imaging in cancer diagnosis lies in the number of expression of tumor cell LHRH-R, as well as specificity level of its combination with LHRH. This study employs immunohistochemical method to detect LHRH-R expression level in tumor cells and normal tissues; pre-tinning to directly label LHRH, measuring and calculating labeling rate and radiochemical purity of labeled product, observing its stability in vivo and in vitro, and selecting labeling method and its optimal condition with higher radiochemical purity and sound stability through comparative analysis. Normal mice were used for live animal experiment to detect the expression levels in tissues and organs of normal live animals as well as metabolism of radio-labeled LHRH in vivo of live animals. The value of LHRH-R expression in tumor cells and LHRH receptor imaging in targeting diagnosis of tumors provided theoretical and experimental basis for further study.

Materials and methods

LHRH-R expression in tumor and normal tissue

Specimen collection. Paraffin specimen of related tumors were collected. Criteria of inclusion were as follows: i) Tissue blocks were fixed in 10% formalin and embedded in paraffin in a routine manner, and were sliced into several sections at the thickness of 4 µm; ii) none of the patients had received preoperative radiotherapy and chemotherapy; iii) surgical treatment were radical resection and tumor-free margins exist; and iv) the pathological diagnosis complied with classification criteria of WHO lung cancer histology in 1998, which did not include small cell lung cancer, carcinoid tumors and metastatic cancer. Qualified cancer tissue samples in the group were 93 cases, including 10 cases of prostate cancer, 20 cases of breast cancer, 20 cases of endometrial cancer, 23 cases of liver cancer and 20 cases of lung cancer.

Treatment of specimens

Treatment of slides. Fresh slides were immersed in cleaning solution for 24 h, rinsed with clean water for 2 h, washed 3 times with distilled water, and place in oven at 37°C after clean rinsing, soaked in 1:10 poly-L-lysine for approximately 10 sec, drained and placed in oven at 37°C. All the specimens were confirmed by pathology.

Treatment of specimen slices. The specimens were fixed in 10% formalin and embedded in paraffin, and were sliced into several pieces at 4 µm, and placed at 37°C incubator for 2 h for standby application.

Control setting. With phosphate-buffered saline (PBS) substituting primary antibody as negative control, the remaining steps were unchanged.

Immunohistochemical staining procedure. Paraffin sections were dewaxed in xylene and hydrated with graded alcohol, and then were washed with PBS (pH 7.4) 3 times, each time for 3 min. The sections were placed in freshly-prepared boiled (pH 6.0) citrate buffer for antigen retrieval. To each slice was added a drop of 3% hydrogen peroxide solution, and incubated at room temperature for 15 min, so as to block endogenous peroxidase and was washed with PBS 3 times, each time for 3 min.

After tossing PBS solution, to each slice was added with a drop of primary antibody (1:200 dilution), overnight at 4°C. Blank control used primary antibody as negative control, the remaining steps were unchanged.

After removing PBS solution, each slice was added with a drop of anti-rabbit HRP polymer (reagent B), and incubated at room temperature for 20 min. PBS rinsing followed 3 times, each time for 3 min.

After removing PBS solution, each slice was added a drop of 3% hydrogen peroxide solution, and incubated at room temperature for 30 min. PBS rinsing was 3 times, each time for 3 min. After removing PBS solution, each slice was added a drop of anti-rabbit HRP polymer (reagent B), and incubated at room temperature for 30 min. PBS rinsing was 3 times, each time for 3 min.

After removing PBS solution, each slice was added with a drop of freshly-prepared DAB coloration liquid, and observed under the microscope for 3 to 5 min, positive coloration is brown, using tap water for rinsing to terminate the coloration.

The slices were rinsed with distilled water and redyed with hematoxylin using 0.1% hydrochloric acid to differentiate and tap water to rinse the slices, and then stained blue. Employing graded alcohol to dehydrate, xylene for transparency, and mounting with neutral gum. Elivision reagent kit contained the reagents A and B.

Immunohistochemical results. LHRH-R positive coloration occurred mainly on cancer cell cytoplasm taking on
brown particles. Immunohistochemistry using semi-quantitative scoring method for determination, i.e., the overall score was calculated according to staining intensity and the percentage of number of positive cells in total number of tumor cells.

Rating according to the degree of positive staining: 0, no coloring, consistent with the background color; 1, pale yellow, slightly higher than the background color; 2, claybank, significantly higher than the background color; and 3, brown.

Rating according to the percentage of positive cells: 0, negative; 1, <10%; 2, 11-50%; 3, 51-75%; and 4, >75%.

The product of the two was calculated to determine the positive result: 0-2 indicated negative (-); 3-4 indicated weak positive (+); 5-8 indicated moderately positive (++; and 9-12 indicated strong positive (+++).

Using pre-tinning $^{99m}$Tc to directly label LHRH
Labeling method. After adding 0.1 ml of sodium gluconate (0.3 mol/l, dissolved by PBS buffer), 0.05 ml of stannous chloride that was dissolved by concentrated hydrochloric acid (40 mg/ml), 0.1 ml of $^{99m}$TcO$_4^-$ physiological saline eluent, and 0.1 ml of PBS buffer into the reaction tube, it was placed at room temperature for 10 min after being mixed in vortex mixer. NaOH was used to set pH at 3.0, and 10 µg LHRH was added. Placing it in a constant temperature water bath at 40°C for 1 h. The labeling was completed.

Changing labeling conditions. i) Labeling under the circumstances of using sodium gluconate as the complexing agent and without the complexing agent, respectively; ii) dosage of stannous chloride and the pH of the reaction system were changed, respectively, changing, dosage of SnCl$_2$ increased from 20 to 60 mg/ml, pH of the reaction increased from 2.0 to 4.0; and iii) the reaction temperature increased from 25 to 50°C.

Stability in vitro. $^{99m}$Tc-LHRH was added to fresh saline and normal human plasma (37°C), respectively, and mixed, and then radiochemical purity was measured at 30 min, 1 h; 90 min, 2, 3 and 4 h.

Determination of radiochemical purity. No. 1 Xinhua chromatography paper was used as stationary phase, and saline as mobile phase to measure radiochemical purity (development system I); no. 1 Xinhua chromatography paper soaked in 2.5% bovine serum albumin (BSA) was adopted as stationary phase, and ethanol developing solvent:ammonia:water = 2:1:5 to measure colloid (development system II), and GC-300γ counter was employed to measure radioactivity.

Result criteria. In development system I, the free $^{99m}$Tc went to the leading edge with the expansion of solvent (RF = 1.0), while the colloid and marker remained at the original point (RF = 0); in eluent II, the colloid was maintained at the origin (RF = 0), while the free $^{99m}$Tc and markers migrated to the forefront along with the solvent (RF = 1.0); eluent I and II system could completely distinguish $^{99m}$Tc-LHRH, colloids and free $^{99m}$Tc in this study. The finally obtained radiochemical purity of $^{99m}$Tc-LHRH exceeded 90%, and colloid content was <10% indicating successful labeling.

$^{99m}$Tc-LHRH receptor binding in vitro
Preparation of rat pituitary cell membrane protein. The whole experiment process was set in an ice bath. Pituitary was quickly removed after the sacrifice of Sprague-Dawley (SD) rats, and rinsed in an ice bath of pH 7.4 10 mmol/l Tris-HCl (containing 0.5 mmol/l PMSF, adding PMSF, 1.2 mmol/l MgCl$_2$, 0.01 mmol/l EDTA-Na$_2$ before using) twice to remove blood.

The pituitary was set inside the electric glass homogenizer for homogenizing after the rinsing (2,000 rpm, 10 sec x 3 times), and centrifuged at 4°C (2,000 x g, 5 min). After discarding the sediment, the supernatant was centrifuged again at 4°C (20,000 x g, 20 min), then supernatant was discarded and the sediment was rinsed 10 times the volume of Tris-HCl twice (20,000 x g, 20 min), thus, the rat pituitary cell membrane protein was attained. A small amount of Tris-HCl suspension was added. BCA protein assay kit was employed to measure absorbance values at 562 nm on a microplate reader. According to the standard curve scheme membrane protein concentration, protein concentration was adjusted to 2 mg/ml, and placed at -70°C for cryopreservation separately.

Receptor assay. The 12x75 mm test tube was taken and prepared with 1% BSA (Tris-HCl, pH 7.4 preparation) before using (BSA was added to the tube, and kept overnight at 4°C, and then BSA was emptied, and the tube was inverted for standby application).

All the tubes were added with 50 µl of rat pituitary membrane proteins (100 µg), including sub-unit binding tube (TB) and nonspecific binding (NSB) tubes, then followed by 2, 5, 10, 20, 30, 40 and 50 µl of $^{99m}$Tc-LHRH. NSB tubes were added with 2 µg gonadorelin (prepared with Tris-HCl to ensure there were much more gonadorelin than $^{99m}$Tc-LHRH in NSB tube), and finally the reaction volume was complemented to 150 µl with Tris-HCl (this buffer was made by adding 0.2% BSA to protein-extraction buffer, pH 7.4).

The tubes were incubated at 37°C set oscillator for 1 h and were added 1 ml precooled Tris-HCl at 4°C after the removal. The reaction was quenched by mixing. The left reactant was collected on glass fiber filter paper 49 with multi-point cell harvester (ZT-II type), and the reaction tube was added Tris-HCl and washed 3 times (1 ml/time), and finally 1 ml 5% TCA filter paper was placed in the test tubes. Measurement of radioactivity of each tube was counted with $^γ$ tube immune counter, as well as TB and NSB.

Measured results employed SB/FREE as the vertical axis, and the amount of specific binding SB as the abscissa, and Scatchard plot, equilibrium dissociation constant KD was attained, SB (dpm) = TB (dpm) - NSB (dpm), unbound $^{99m}$Tc-LHRH F (dpm) = added $^{99m}$Tc-LHRH T (dpm) - total binding TB (dpm).

LHRH metabolisms in vivo
Preparation of $^{125}$I-LHRH.
Chloramine-T method to label LHRH. 4 mCi $^{125}$I/0.4 µl, LHRH 10 µg/10 µl, and 50 µg chloramine-T were added into Eppendorf (EP) tube successively. The mixed solution was left to react for 90 sec (during the reaction, the solution was shaken on the vortex mixer), then 100 µg Na$_2$S$_2$O$_3$ was added and the solution was mixed on the vortex mixer, finally the reaction was terminated. The labeling was completed.
Production of Sepahdex G-25 gel column. After the G-25 gel particles were immersed into pure water for 24 h, they were added into glass acid buret containing glass fiber at the bottom. After the air was exhausted, using 10 column volumes of PBS buffer to rinse slowly, then 10 ml 1%. The BSA saturated gel column was added.

Separation and purification of $^{125}$I-LHRH. $^{125}$I-LHRH was added into Sepahdex G-25 gel column, and the reaction tube was washed with PBS 2 times, each time 100 µl, gel column was also added, and was rinsed slowly with PBS. Dripping speed was adjusted to 6 drops/min, and leaching liquid solution was collected (1 ml/tube). Employing paper chromatography to measure its radiochemical purity, respectively (no. 1 Xinhua paper chromatography paper was used as stationary phase and the normal saline as the mobile phase, γ counter was used to measure radioactivity count). The highest radiochemical purity was taken for further experiments.

Animal experiments. Forty-five healthy male mice aged ~23 weeks and weighing 20±2 g were divided into 9 groups according to random number table, with each group including 5. The 54.1 mCi/100 µl $^{125}$I-LHRH was injected into tail vein. A group of mice was taken, respectively, 15 and 30 min, 1, 2, 4, 6, 24, 48 and 72 h after the injection. Blood was collected from picked eyeballs. Using stem dislocation to sacrifice the mice and then they were dissected, major organs (heart, lungs, liver, spleen, kidney, muscle, bone, brain, small intestine and stomach) were taken and weighed. γ counter was used to measure radioactivity count of blood and organs, and unit mass of tissue percentage radioactivity uptake dose rate (%, ID/g) was calculated (per gram of tissue radioactivity count/total radioactivity injected into mice count x 100%).

Statistical analysis. Data are presented as mean ± standard deviation (mean ± SD). SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for Fisher's exact test. P<0.05 was considered to indicate a statistically significant difference.

Results

Immunohistochemical staining. Immunohistochemistry of specimens had good stain specificity. Pale background or blank background. Positive product of immunoreaction was brown. As contrast was obvious, it was easy to confirm. Immunohistochemical staining results of LHRH in different tissues are shown in Table I and Fig. 1A-H.
Labeling rate and radiochemical purity of $^{99m}$Tc-LHRH. In development system I, the free $^{99m}$Tc went to the leading edge with the expansion of solvent (RF = 1.0), while the colloid and marker remained at the original point (RF = 0); in eluent II, the colloid was maintained at the origin (RF = 0), while the free $^{99m}$Tc and markers migrated to the forefront along with the solvent (RF = 1.0); eluent I and II system could completely distinguish $^{99m}$Tc-LHRH, colloids and free $^{99m}$Tc in this study. The highest LHRH labeling rate by employing direct method of no. 1 Xinhua paper chromatographic hit (98.7±0.93%); the highest radiochemical purity reached 95.2±1.02% (Fig. 2A-C).

Impact of complexing agent. In the case of no complexing agent was used, the presence of white sediment in the reaction system signified unsuccessful labeling. With 0.1 ml of sodium gluconate (0.3 mol/l) as complexing agent, when pH value stood at 3.0, the radiochemical purity was up to 95.2±1.02%, colloid content in the labeling reaction was <5%.

Impact of SnCl$_2$·2H$_2$O dosage and pH value on radiochemical purity. In case that other labeling conditions (volume of $^{99m}$Tc eluent, reaction temperature, peptide dosage) remained the same, SnCl$_2$·2H$_2$O and pH value were changed, resulting radiochemical purity is shown in Table II.

Effect of reaction temperature. In case that other labeling conditions (volume of $^{99m}$Tc eluent, SnCl$_2$·2H$_2$O dosage, pH value and peptide dosage) remained the same, the reaction temperature was changed. At 25°C, the radiochemical purity was 32.6±1.9%, at 40°C, it was 91.6±3.5%, at 50°C, it was 57.8±1.9%.

Stability in vitro of labeled product. Within 3 h, reactants maintained a high radiochemical purity and declined sharply after 3 h (Table III and Fig. 3).

In vitro binding of $^{99m}$Tc-LHRH receptor. Receptor saturation analysis showed that total combination increased with $^{99m}$Tc-LHRH and specific binding rose quickly at the outset. When $^{99m}$Tc-LHRH was increased to a certain amount, the curve flattened and stopped increasing, and showed saturation trend, indicating that the receptor has been absorbed by $^{99m}$Tc-LHRH. NSB increased linearly with $^{99m}$Tc-LHRH, showing no saturation trend (Table IV and Fig. 4A). With B/F as the vertical axis, the binding capacity as the abscissa, and figure was drawn by Scatchard plot, RT = 23.2174 pmol, KD = 0.4348 nmol (Fig. 4B).

Distribution of $^{125}$I-LHRH in normal mice. Distribution result in normal mice can be seen in Fig. 5. $^{125}$I-LHRH was injected into the mouse tail vein, 15 min later, blood radioactivity was
up to 3.09% ID/g, which was then quickly cleared, and 4 h later, it reduced to <1% ID/g; radioactivity distribution in kidneys was high, and peaked at 9.24% ID/g after 15 min, then decreased rapidly, and 1 h later, it decreased to 1.35% ID/g, followed by a rapid rise to 5.85% ID/g again, and then gradually cleared away; liver also had a high radioactivity uptake, and 15 min later, it reached 5.79% ID/g, which may be associated with blood perfusion and nonspecific uptake, and then was removed quickly; gastrointestinal uptake was relatively high at the outset, and peaked 2 h later at 6.69% ID/g and 4.50% ID/g, respectively. Radioactivity in heart, lung, spleen, brain, bones and muscles decreased over time.

Discussion

In this study, by using immunohistochemistry assay, LHRH positive rate in 23 cases of HCC was 82.61%, and positive rate of corresponding normal tissues was 15%; positive rate of 20 cases of breast cancer reached 95%, and positive rate of the corresponding normal tissues was 20%; positive rate of 10 cases of prostate cancer, was 70%, while positive rate of corresponding normal tissues was 40%; positive rate of 20 cases of lung cancer was 85%, and positive rate of corresponding normal tissues reached 15.79%; positive rate of 20 cases of endometrial cancer was up to 80%, and positive rate of corresponding normal tissues provided the foundation for using LHRH body imaging.

Peptide radioactive agent has many advantages: Ligand peptide is chemically synthesized, which overcome the heterogeneity problem, and almost does not incur human immunogenicity response; its low molecular weight makes

Table II. Impact of SnCl₂·H₂O dosage and pH value on radiochemical purity (%).

| SnCl₂·H₂O (µg) | pH value |
|----------------|----------|
|                | 2        | 2.5     | 3        | 3.5      | 4        |
| 1,500          | 65.1±3.2 | 57.6±1.6| 58.1±1.3 | 60.4±2.1 | 49.3±1.3 |
| 2,000          | 83.8±0.9 | 83.5±2.7| 93.9±1.0 | 88.4±0.8 | 72.4±3.7 |
| 2,500          | 75.1±2.4 | 82.8±2.0| 64.9±4.0 | 60.7±1.1 |           |

*White sediment appeared.

Table III. Stability in vitro of labeled product.

| Degrees         | 0 h     | 0.5 h   | 1 h     | 1.5 h    | 2 h     | 3 h     | 4 h     |
|-----------------|---------|---------|---------|----------|---------|---------|---------|
| 25˚C saline     | 95.2±1.023 | 88.7±2.472 | 88.1±3.556 | 88.1±3.044 | 86.2±2.434 | 87.3±2.055 | 71.2±2.575 |
| 37˚C saline     | 95.2±1.023 | 95.0±1.466 | 94.2±1.563 | 91.7±0.634 | 89.0±2.677 | 85.8±10.537 | 48.2±2.423 |
| 37˚C human serum| 95.2±1.023 | 95.1±1.359 | 94.4±0.249 | 91.4±1.178 | 86.4±1.744 | 74.6±2.439 | 23.0±3.146 |

Table IV. Experiment data of in vitro binding of ⁹⁹ᵐTc-LHRH receptor.

| Protein nos. (µl) | Total count (T) | Total binding count (TB) | Count of non-specific (NSB) | Count of specific binding (SB) | Corresponding LHRH amount of binding (pmol) | B/F     |
|------------------|-----------------|--------------------------|-----------------------------|-------------------------------|---------------------------------------------|---------|
| 2                | 12,742          | 1,088                    | 587                         | 501                           | 3.9318                                      | 0.04299 |
| 5                | 31,855          | 1,795                    | 714                         | 1,081                         | 8.484                                       | 0.03596 |
| 10               | 63,710          | 2,465                    | 923                         | 1,542                         | 12.1016                                    | 0.02518 |
| 20               | 127,420         | 3,462                    | 1,367                       | 2,095                         | 16.4416                                    | 0.0169  |
| 30               | 191,130         | 4,056                    | 1,678                       | 2,378                         | 18.6625                                    | 0.01271 |
| 40               | 244,840         | 4,575                    | 2,176                       | 2,399                         | 18.8274                                    | 0.009985|
| 50               | 318,550         | 4,835                    | 2,486                       | 2,349                         | 18.435                                     | 0.007488|

LHRH, luteinizing hormone-releasing hormone.
it easy for peptides to get through physiological barrier and penetrate tissues, and it can reach target and clear away blood quickly, which helps to reduce radioactive background and improve tumor/non-tumor (T/NT) ratio (43), therefore high-contrast tumor imaging can be obtained in a relatively short time.

LHRH-R shows high expression in a variety of tumor cells. Using appropriate radionuclide to label LHRH analogues is conducive to early diagnosis and treatment of related cancer. There are now a number of radionuclides for marking LHRH analogues (44).

Chloramine-T methods most commonly used to label LHRH with iodine. Radioactive iodine (such as $^{123}$I/$^{125}$I/$^{131}$I) is labeled on the 5th tyrosine of LHRH. The labeling process is simple with mild reaction conditions (45). After the labeling, separation and purification of free iodine and iodine binding peptides must be conducted, which takes a long time, and because the iodine connected to tyrosine is easy to fall off in the body and $^{123}$I is expensive, the application of iodine-labeled LHRH is rare (46,47).

$^{18}$F labeled-LHRH mostly uses indirect labeling method. $^{18}$F often causes deformation to LHRH molecular structure, thereby affecting its biological activity, so labeled prosthetic groups or the so-called bi-functional ligands are widely used to connect radionuclides and biologically active molecules (31). However, such labeling methods often have various radioactive steps and long duration (36).

Relative to other radionuclides, $^{99m}$Tc possesses moderate energy, and its gamma-ray energy is 140 keV, which is readily available and of low cost. It can be easily obtained through $^{99}$Mo-$^{99m}$Tc generator, which is currently the most commonly used clinical radionuclide. Main methods of using $^{99m}$Tc to label polypeptides are as follows: i) Indirect labeling method carried out by chelating agent; ii) direct reduction method; and iii) using biotin and avidin system to label (48). Currently, modified Schwartz method and pre-tinning method are frequently used (31). In this study, pre-tinning was adopted to directly label LHRH, and adding sodium gluconate in the reaction system as a complexing agent, in order to prevent the formation of Sn-colloid, Tc-colloid and Sn-Tc colloid. By altering pH value of reaction system, reaction temperature and the amount of reducing agent, labeling rate and radiochemical purity of $^{99m}$Tc-LHRH under different conditions were compared, the resulting labeling rate of optimal labeling method was 97.9-100.0%, radiochemical purity was up to 93.9-96.4%, and colloid content in the labeling reaction was <5%. The method is simple with the high radiochemical purity of the resulting product. Although 3 h after being placed in 37°C human serum, the radiochemical purity dropped to ~75%, but 2 h later, it was still nearly 90%. Given that in the present clinical study, imaging often occurs 2 h after the introducing imaging agent, $^{99m}$Tc-LHRH obtained by this method may become imaging agent for LHRH-R positive tumor receptors.

Moreover, $^{99m}$Tc-LHRH has receptor binding characteristics, which is a basic condition for receptor imaging agent. In receptor analysis, plenty of gonadorelin was added into NSB, this peptide served as LHRH antagonist, three receptor analysis revealed radioactivity counting measurement of each NSB tube took on linear relationship with corresponding $^{99m}$Tc-LHRH, and all exceeded 0.90, suggesting that at this time substantial amount of gonadorelin membrane proteins occupied LHRH-R sites in pituitary, measurement of each tube took on linear relation, which indicated that these counts were combination of $^{99m}$Tc-LHRH, miscellaneous proteins in the cell membrane, and test wall rather than count formed by its binding to the receptor, i.e., NSB. In TB tube, because there was no LHRH, radioactive counts of each tube consisted of two parts, one part of count was formed by $^{99m}$Tc-LHRH binding to its receptor (i.e., SB), and one part was the NSB count of $^{99m}$Tc-LHRH, therefore SB
was obtained through TB-NSB. The count of each TB tube increased rapidly as the amount of $^{99m}$Tc-LHRH increased, indicating that the receptor had a surplus, then the count gradually increased, indicating that the receptor binding was basically complete, and the count increased simply because of the NSB.

The SB obtained showed similar curves with TB, and although TB curve later rose slowly, it still increased as $^{99m}$Tc-LHRH went up, but at certain time SB curve flattened, showing that receptors were saturated. Usually, affinity between receptor and ligand is bound by $10^{-9}$, KD $\geq 10^{-9}$ indicating the low affinity between receptor and ligand, the bigger the KD, the lower the affinity, and KD $\leq 10^{-9}$ indicated high affinity between ligand and receptor, which belongs to high affinity ligand, the smaller the KD, the higher the affinity. In this study, by external cell membrane protein receptor saturation binding experiment, it was confirmed that $^{99m}$Tc-LHRH could be suppressed by LHRH, RT = 23.2174 pmol, KD = 0.4348 nmol, which indicated the labeled $^{99m}$Tc-LHRH was the low-capacity and high-affinity ligand of the receptor. Affinity was similar to 0.4792 nmol, KD value of carcinoembryonic cell receptor obtained by predecessor who used $^{99m}$Tc-LHRH, but lower than 10 nmol, KD value obtained by Barda et al who analyzed the combination of external binding between $^{99m}$Tc-LHRH and receptor in rat pituitary cell membrane (19). Probably differences in tissue origin of receptor specimens and preparation methods, as well as employed LHRH structure caused the figure discrepancy. Receptor analysis demonstrated that $^{99m}$Tc-LHRH was the receptor's high affinity ligand, i.e., $^{99m}$Tc-LHRH and unlabeled LHRH had a high affinity for its receptor, and its biological activity and receptor affinity was unaffected, which also provided an experimental basis for $^{99m}$Tc-LHRH as receptor imaging agent for LHRH-R positive tumors.

In vivo metabolism experiment of $^{125}$I-LHRH in a live animal showed that after intravenous injection of $^{125}$I-LHRH in the tail, blood radioactivity of the mice reached a maximum of 3.09% ID/g after 15 min, then quickly faded away, and 4 h later the figure was <1% ID/g, which helped to reduce the background and increase the target/non-target tissue radioactivity ratio; distribution of radioactivity in kidneys was very high, and peaked at 9.24% ID/g after 15 min, but followed by a rapid decrease, and after 1 h, the figure was 1.35% ID/g, and then again rapidly increased to 5.85% ID/g, then gradually faded away. The first peak indicated the high perfusion of kidney tissue after the tail vein was injected with $^{125}$I-LHRH, and the second peak suggested that the imaging agent was mainly excreted through the urinary system, which was conducive to the detection of chest and abdominal tumor; liver and lungs also had a higher uptake, which peaked at 15 min and may be associated with blood perfusion and non-specific uptake, followed by rapid clearance. Immunohistochemical results showed normal liver and lung tissue has a certain amount of receptor expression, but receptor expression in the tumor was much higher than that in normal tissue, which was conducive to the detection of liver and lung tumors; gastrointestinal radioactivity uptake was relatively high at the outset, peaked at 2 h, which was 6.69% ID/g and 4.50% ID/g, respectively. On one hand, it showed that there were certain amounts of LHRH-R expression in gastrointestinal tissue; on the other hand, it showed that part of marker was excreted from the digestive tract, hence it was not suitable for the detection of gastrointestinal cancer. $^{125}$I-LHRH in heart and spleen mainly took on blood pool type distribution, and its concentration decreased with clearance of the blood background. Brain, bone and muscle radioactivity decreased over time.

In conclusion, positive rate of LHRH-R expression in human liver, lung, breast, prostate, endometrial tumors is significantly higher than that in adjacent normal tissue, and difference in expression intensity compared with the normal control group is statistically significant. Studies have shown that this labeling method is fast and easy, and the resulting radiochemical purity of the labeled product is relatively high, which does not call for further purification and has good stability. $^{99m}$Tc-LHRH labeled by this method has high affinity in its binding with its receptor and its biological activity and receptor affinity is not significantly affected. Radionuclide labeled LHRH has an ideal kinetics performance in the body of animal, which holds promise for an imaging agent of clinically practical value of LHRH receptor.

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