Original Article

Anti-GPC3 single-chain scFv antibody acts as an agent for radio-immunoimaging in diagnosing hepatocellular carcinoma

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Abstract: Glypican-3 (GPC3) over-expresses in hepatocellular carcinoma (HCC), but not expresses or under-expresses in normal adult hepatocytes. Therefore, GPC3 acts as a potential target for diagnosis and treatment of HCC. This study aimed to conduct radio-immunoimaging using GPC3 as a target in order, and to explore its potential for diagnosing and treating HCC. Humanized single-chain antibody scFv for HCC was established using phage antibody library. E.coli HB2151 was infected with recombinant phage antibodies that are considered to be strongly positive by phage ELISA. Then, the soluble antibodies were obtained post IPTG induction. Soluble antibodies were detected using SDS-PAGE assay. Anti-GPC3 single-chain antibodies were labeled using ¹³¹I, and then the distribution of radioactive markers in nude mice were analyzed in vivo by radio-immunoimaging. The results indicated that the size of soluble scFv products was 30 kD after purifying anti-GPC3 scFv antibodies that are successfully screened from phage antibody library. Anti-GPC3 single-chain antibodies were specifically bind to HCC cells. The ratios of radioactive tumor/blood and tumor/muscle for ¹³¹I labeled anti-GPC3 monoclonal antibodies were increased gradually, achieving the highest at 48 h. Radio-immunoimaging showed that the radioactive uptake of tumor sites remained the strongest at 48 h, and the ratio of target to non-target was the highest. In conclusion, the established Anti-GPC3 scFv had the potential to become an agent for radio-immunoimaging in diagnosing HCC and act as a targeted antibody for further radio-immunotherapy of HCC.

Keywords: Hepatocellular carcinoma, GPC3, phage display technology, single-chain antibody, radio-immunoimaging

Introduction

Presently, surgical resection remains to be the most important treatment for hepatocellular carcinoma (HCC). This treatment provides satisfactory treatment effects for patients with early-stage HCC, however remains poor in patients with advanced HCC. In early stage of HCC, the clinical symptoms are not typical, and most of the patients present advanced stage of HCC during diagnosis and lose the surgical opportunity. Improving the detective rate of early-stage HCC remains of great significance for prognosis of HCC patients [1, 2]. Nowadays, there is still no good diagnostic method for screening or detecting the early-stage HCC.

Diagnosis of HCC using serum alpha-fetoprotein shows poor sensitivity, and there is usually no increase in the alpha-fetoprotein for well differentiated small HCC. Increase of serum alpha-fetoprotein can be detected during the inflammations, chronic viral hepatitis, and etc. [3, 4]. Whereas conventional ultrasound and CT scan are sometimes difficult for diagnosing the early stage HCC less than 1 cm and isometric tumors. Presently, there are a variety of non-surgical methods designed for treating HCC, such as transcatheter arterial chemoembolization, hepatic artery or portal vein perfusion chemotherapy, and etc. However, these methods could easily cause the toxic side effects administrating together with the chemotherapy, such as liver failure and severe bone marrow depression, forcing to interrupt or abandon of the treatment.

The targeted diagnosis and treatment of tumors have attracted more and more attention. Meanwhile, the targeted diagnosis and treat-
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ment also have the advantages of higher specificity to target organs or cells and the lower damage to normal cells. Therefore, investigations for the target tumor markers have important clinical significance in diagnosing, treating and therapeutic monitoring of tumors. In recent years, non-invasive molecular imaging and therapeutic techniques have received considerable attention in diagnosis and treatment of solid tumors. Small-molecule antibodies binding to specific antigens on surface of cells have demonstrated higher specificity and affinity [5]. Moreover, the natural distribution of drugs in body alters after labeling the radionuclides, which leads to drug enrichment in tumor area. This in turn exerts its specific killing effect, which is superior to radiotherapy and chemotherapy due to characteristic of selective killing of HCC cells. Antibody plays an important role in radio-immunoimaging and treatment. Single-chain antibody fragment scFv (single-chain antibody fragment), as a humanized antibody, is characterized by lower molecular weight, therefore, which has lower heterogeneity and doesn’t generate human anti-mouse antibody (HAMA). Furthermore, single-chain antibody fragment scFv also has characteristics of strong penetrability and rapid serum clearance [6, 7]. Humanized single-chain antibody fragment scFv obtained by phage display technology has the ability to specifically bind to target cells. The above effect of fragment scFv in turn effectively improves tissue penetration of antibodies and reduces immunogenic response. Meanwhile, fragment scFv also decreases half-life of blood and provides effective ratio of target tissue to non-target tissue, making it an ideal antibody for radio-immuno-diagnosis and treatment [8].

Glypican-3 (GPC3) is a member of phosphatidylinositol family with a molecular weight of 70 kD, and anchors to the cell surface via glycosylphosphatidylinositol (GPI) [9, 10]. Glypican-3 expression is closely related to the occurrence, development and prognosis of HCC [11]. GPC3 protein has proven to be highly expressed in HCC, while lowly expresses or never expresses in normal liver tissues. GPC3 protein has certain specificity and sensitivity, making it to be a popular and potential target for diagnosis and specific treatment of early-stage HCC [12]. Over-expression of GPC3 is closely related to the occurrence and development of HCC [13]. There were very few studies evaluate the immunotherapy using GPC3 targeting approaches, such as humanized anti-GPC3 cytotoxic antibody, peptide vaccine and immunotoxin therapy [14, 15].

Therefore, this study attempted to construct a phage single-chain antibody library for humanized HCC, and screen anti-GPC3 specific single-chain antibody fragment scFv. Radioactive iodine was labeled using the screened recombinant and purified single-chain antibodies. In turn, the antibodies were injected into HCC-bearing nude mice for radio-immunoimaging to observe the target specificity of anti-GPC3 single-chain antibody for HCC. This study aimed to clarify the effects of radiopharmaceutical-labeled anti-GPC3 single-chain antibody on diagnosing and treating the HCC.

Materials and methods

Construction of humanized single-chain antibody fragment scFv

Construction of humanized single-chain antibody for HCC was conducted by referring to the methods in the previous literatures [16, 17]. To separate the lymphocytes, 100 ml of peripheral blood was collected from 20 patients who were pathologically diagnosed as HCC. All specimens were obtained by the approval of Ethics Committee of the hospital where the authors worked in. All patients provided signed informed consent forms.

Total RNA was extracted from self-isolated lymph node cells using TRIzol (Invitrogen, USA) reagent. The first chain of complementary DNA (cDNA) was synthesized using reverse transcriptional real-time PCR (RT-PCR). With cDNA as the template, the defined primers were used to amplify $V_H$ and $V_L$ gene fragments, respectively. $V_H$ and $V_L$ gene fragments were extended to connect to peptides $V_H$-linker and $V_L$-linker. Meanwhile, the scFv was synthesized using SOE-PCR. Single-chain antibody genes (scFv Gene) were digested with restriction endonucleases, including SfiI and NotI (BioTeke Company Beijing, China). Then, scFv Gene underwent the ligation reaction with digested phage vector pCANTAB-5E (New England Biolabs, New England). After purification of the ligation products, electro-transformation was conducted for Ecoli TG1 (New England Biolabs,
New England), following by coating of LB plate. Then, the transformation efficacy was determined using pUC19 standard plasmid. Gradi-
ent dilution was prepared for the antibody library. Total of 1 μl antibody library solution was used to infect 100 μl of E.coli, and then SOBAG plate was coated. The number of bacterial colonies was counted to calculate the colony-forming units. Clones with positive PCR were identified with double digestion using SfiI and NotI. Subsequently, the clones underwent electrophoresis on 1.0% agarose gel for products to identify whether there was any release of target fragments.

**Screening of phage antibody library using HCC cell line HepG2**

Phage antibody library was absorbed using normal hepatocyte HL-7702 to remove non-specific antibodies. After that, 3 rounds of screening and enrichment of antibody library were conducted by absorption, elution and amplification with HepG2 cells (1×10⁷). The solution was then diluted using GPC3 antigens to concentration of 10 μg/ml, followed by coating at 4°C and maintained overnight. On the next day, the solution was sealed using 2% BSA/PBS at 37°C for 2 h, followed by incubation using 100 μl of antibody library screened by HL-7702 cells for 1 h. The solution was washed using phosphate buffered saline Tween-20 (PBST) to remove any unbound or weakly bound phages. Phages were recovered using 100 ml of elution. Three rounds of screening were conducted. The screened and enriched phages were used to infect E.coli TG1, and then the SOBAG agar plate was coated. The number of bacterial colonies was then counted. The harvesting rate of the antibody library was calculated after corresponding enrichment of similar operations.

**Screening of phage antibody library using GPC3 antigen**

The screened and enriched phages were used to infect Escherichia coli TG1. The bacterial solution was used to coat SOBAG plate, which was then cultured at 30°C overnight. On the next day, the bacterial colonies were inoculated into a 96-well plate to prepare single-chain phage antibodies. Enzyme-linked plate was coated with GPC3 antigens, and phage antibodies were added after sealing. The plate was washed after incubation overnight. HRP/anti-M13 antibody was used as a secondary antibody (Abcam, Shanghai, China) (diluted to 1:4000 at 37°C for 1 h), which then washed with PBST. TMB was then added for the coloration. The absorbance value (OD value) was measured at 450 nm post adding the stop buffer. OD value in the experimental group greater than that of control group was used as positive wells.

**Phage antibody library detected using ELISA**

E.coli HB2151 was infected with recombinant phage antibodies that were detected as strong positive by ELISA, which was then streak-inoculated on a SOBAG-N plate overnight. The 2× YT-AG culture medium was added into the bacterial solution and cultured till the logarithmic growth phase, and then IPTG was added. Bacteria were collected by centrifugation post violent shaking for 4 h and 6 h, respectively. The bacteria were re-suspended and PBS buffer was added, followed by rapidly freezing in liquid nitrogen for 30 min. Then, the solution was thawed at 37°C. The bacteria after repeated freezing and thawing for 3 times were broken. The obtained bacterial solution was then centrifuged and the supernatant was collected to express soluble scFv.

**scFv identification using SDS-PAGE and western blot assay**

SDS-PAGE separation gel (12%) and concentrated gel (5%) were prepared, and then were subjected to SDS-PAGE assay (Beyotime Company, China). Total of 20 μl scFv with soluble expression was added into the loading buffer, and then stained using Coomassie R250. Purification column of bacterial solution successfully expressed soluble scFv by SDS-PAGE was purified. Identification was conducted using western blotting assay (Beyotime Biotech., Company, China). Horse radish peroxidase (HRP)-mouse anti-E-tag was used as the primary antibody, and HRP-anti-mouse antibody was used as the secondary antibody. Chemiluminescence was conducted, and then development and fixation were conducted.

**Cellular ELISA and immuno-cytochemistry**

Ovarian cancer cell line (SKOV3) and HCC cell line (HepG2) were cultured to prepare a suspension and then added into a 96-well plate for
ELISA assay. The suspension was washed with PBS thrice (5 min each time). The suspension was fixed using 0.25% glutaraldehyde for 10 min, and then sealed for 1 h. After that, the suspension was washed using PBST and PBS for 3 times (5 min each time). Purified positive recombinant antibodies were added as the primary antibody, while HRP/Anti-E tag was used as the secondary antibody to incubate suspension (ZSGB-BIO, China). TMB coloration was performed and the reaction was stopped using the stop buffer. OD values of each well at 450 nm wavelength were measured. The suspension was observed under a light microscopy, and photographs were taken.

Labeling of recombinant antibody scFv with soluble expression using $^{131}$I

$^{131}$I labeling was conducted for purified soluble antibodies with the chloramine-T method. About 100 μl of $^{131}$I at a radioactivity intensity of 55 mCi/ml was mixed well with 520 μl of purified soluble scFv, and 250 μl (1 mg/ml) of chloramine-T solution was added 3 min later. After 1 min of reaction, 500 μl of sodium metabisulfite at a concentration of 2 mg/ml was rapidly added and mixed well. Then, 100 μl of 10% potassium iodide solution was added to stop the labeling. The above solution was purified using Sephadex G200 column (Beijing solarbio science & technology co., ltd, China). The solution was then eluted using PBS. A total of 100 tubes (0.1 ml/tube) of purified markers were collected, and the radioactive count was measured using γ-counter. The first peak with the highest radioactive count was considered as the purified soluble scFv labeled by $^{131}$I. The purified product was filtered using a filter and then stored at 4°C. The labeling rate was calculated. Radiochemical purity was analyzed using paper chromatography. Then, the activity of radioactive ratio was counted, and room-temperature stability and serum stability were measured.

Construction of HCC cell-bearing nude mice models (HepG2)

HCC HepG2 cells were resuscitated and passaged. HepG2 cells at logarithmic growth phase were inoculated to 12 female nude mice aging from 4 weeks to 6 weeks (Animal Center of Chongqing Medical University, Chongqing, China).

Subcutaneous tumors at the left anterior extremity (200 μl/mouse) with a diameter of 1.0 cm from nude mice, aged 6-8 weeks, were used to study the distribution of radioactivity and radio-immunoimaging. The 12 tumor-bearing nude mice were sacrificed at 4 time points of 12 h, 24 h, 48 h and 72 h, respectively. Then, the radioactive counts of various tissues were detected. Moreover, the percentage of injected dose per gram (%ID/g) was calculated.

The animal experiment was approved by the Biomedical Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, Chongqing, China.

Radioactivity distribution of $^{131}$I-scFv in HCC-bearing nude mice

$^{131}$I-labeled anti-GPC3 single-chain antibodies were injected into 12 tumor-bearing nude mice via the tail vein, and 3 nude mice were sacrificed at 12 h, 24 h, 48 h and 72 h, respectively. After anatomy, the eyeball blood was collected, and then the tissue was weighed. After that, the radioactivity counts of tumor, blood, heart, spleen, liver, kidney, muscle, lung, stomach, intestine and brain were measured. Moreover, the percentage of injected dose per gram of tissue was calculated (%ID/g).

SPECT radio-immunoimaging and tomographic image fusion

Three days before radio-immunoimaging, each nude mouse was injected with sodium iodide solution to seal the thyroid glands. Nude mice anesthetized by intraperitoneal injection of chloral hydrate were used on the day of radio-immunoimaging. Purified $^{131}$I-scFv 18.5 MBq was injected to each nude mouse via the tail vein. SPECT imaging (Siemens Symbia T2 SPECT/CT) was conducted at 12 h, 24 h, 48 h and 72 h post the injection. Positive-position static imaging and high-energy collimator were used. The matrix was 256×256 and energy peak was 364 keV. Acquisition was performed for 5 min for each frame. Tomographic fusion imaging was conducted when static imaging showed significant radionuclide uptake at the tumor sites.

Statistical analysis

Statistical analysis was conducted using SPSS19.0 software package (SPSS, Inc., Chicago,
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IL, USA). All data were expressed as means ± standard deviation (mean ± SD). One-way ANOVA was used for comparing the multi-sample mean values. LDS test was performed for multiple comparisons. P<0.05 was considered to be statistically significant.

Results

Construction of phage antibody library

The first chain cDNA was obtained by reverse transcription of total RNA, and then the heavy-chain or light-chain variable region primers were added. The length of the amplified gene fragments of \( V_H \) and \( V_L \) was 370 bp and 350 bp, respectively (Figure 1A). The amplified and purified \( V_H \) and \( V_L \) gene fragments were linked with linker peptide (linker), and the product was about 750 bp in size (Figure 1B). Double enzyme digestion was performed for positive cloning plasmids using Sfil and NotI. 1% agarose gel electrophoresis showed clear bands at 750 marker position, proving that the scFv was successfully recombined (Figure 1C).

ELISA screening for anti-GPC3 positive clones

Fifteen individual bacterial clones were randomly selected from the plate to be added with M13K07 for performing ELISA test. The results showed that 10 monoclonal antibodies were positive with a positive rate of 67% (Figure 2A). Bacterial clones in the same group that had positive reaction for phage ELISA were used. The ELISA identification was performed for soluble antibodies. The results revealed that 10 clones and HepG2 cells showed positive reaction. Moreover, 10 clones were consistent with positive clones in corresponding phage ELISA (Figure 2B).

Analysis of scFv expression using SDS-PAGE

After inducing the expression of E.coli infected with scFv, the supernatant and purified soluble

Figure 1. Recombination and identification of scFv. A. Agarose gel electrophoresis identification of antibody variable range, \( V_H \) and \( V_L \). B. Agarose gel electrophoresis of scFv. C. The results of Sfi and NotI double digestion reaction was positive insert clones.

Figure 2. Screening the anti-GPC3 positive clones using ELISA assay. A. Detection of antigen-positive clones by ELISA. B. The binding specificity and affinity of Anti-GPC3 scFv against HepG2.
scFv were applied for SDS-PAGE. The results revealed that the size of purified soluble scFv product was 30 kD, and soluble scFv was successfully expressed. Positively expressed scFv was used to infect E.coli HB2151. Soluble scFv can be obtained after IPTG induction. Western blot illustrated the significant bands with the molecular weight of 30 kD (Figure 3).

Anti-GPC3 phage antibodies specifically bound to HCC cells

Immunological activity of soluble antibodies was measured using ELISA assay. Variance results showed that immunological activity was 0.47±0.11, 0.18±0.05 and 0.16±0.04, for the HepG2 cell group, SKOV3 cell group and PBS negative control group, respectively. One-way ANOVA showed statistically significant differences among these groups (F=103.934, P=0.000). The multiple comparison also indicated that the immunological activity of HepG2 cell group was higher significantly compared to the in SKOV3 cell group and PBS negative control group (P=0.000).

The immuno-cytochemistry results showed that the anti-GPC3 phage antibodies positively stained HepG2 cells, but phage antibodies did not stain the ovarian cancer cells (Figure 4). Moreover, there was no obvious staining in HepG2 negative cell group, suggesting that anti-GPC3 phage antibodies can specifically bind to HCC cells (Figure 4).

Labeling of scFv using $^{131}$I and its identification

Labeling rate of $^{131}$I-labeled soluble antibodies was 80.2±4.0% by trichloroacetic acid precipitation. Radiochemical purity tested by chromatography was 92.75±3.2%. The intensity of radioactivity was 3.2±0.2 MBq/μg.

Stability of $^{131}$I-scFv at room temperature was shown in Table 1. The radiochemical purity tested by paper chromatography at 4 time points were all greater than 90%, but showed no significant difference (Table 1, P>0.05). The serum stability of $^{131}$I-scFv was shown in Table 2. At 4 time points, the radiochemical purity of the marker was changed with time, but showed no statistically significant difference (Table 2, P>0.05).

Radioactivity distribution of $^{131}$I-scFv in vivo

The results demonstrated that the radioactive count remained the highest at 12 h. As time goes by, radioactive markers were gradually attenuated in vivo. At 4 h, the radioactive ratio of tumor/blood was the highest at 4.68±1.43, and the radioactivity ratio of tumor/muscle was 4.81±1.27 (Table 3).

SPECT/CT radio-immunoimaging of tumor-bearing nude mice

Post the $^{131}$I-labelled anti-GPC3 single-chain antibodies were injected into tumor-bearing nude mice via tail vein, SPECT static imaging was performed (Figure 5). The radioactive uptake of tumor sites in the nude mice was significant after 48 h, while radioactive uptake of other parts of the whole body was low. Region of interest was plotted to compare the T/N value. The T/N value was the highest at 2.95±0.36, suggesting that 48 h was the best time for imaging. Moreover, $^{131}$I labeled anti-GPC3 single-chain specifically enriched to tumor sites in the nude mice, and demonstrated good targetability (Figure 5).

Discussion

Diagnosis and treatment of early-stage HCC, as a hot topic, is difficult in recent years. Clinically, there is still no good method for screening or detecting early-stage HCC. Previous studies have demonstrated that GPC3 highly expresses in HCC, while there is no expression in nor-
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Table 1. Radiochemical purity of $^{131}$I labeled scFv at different time points when laid at room temperature (%, $\bar{x} \pm SD, n=3$)

<table>
<thead>
<tr>
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<th>1 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
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<tbody>
<tr>
<td>Radiochemical purity</td>
<td>93.9±1.3%</td>
<td>94.2±0.4%</td>
<td>92.5±0.69%</td>
<td>91.6±0.90%</td>
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Table 2. Radiochemical purity of $^{131}$I labeled scFv at different time points when kept in serum at 37 °C (%, $\bar{x} \pm SD, n=3$)

<table>
<thead>
<tr>
<th></th>
<th>1 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
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<tbody>
<tr>
<td>Radiochemical purity</td>
<td>93.2±1.2%</td>
<td>92.7±1.0%</td>
<td>91.9±1.0%</td>
<td>90.8±0.7%</td>
</tr>
</tbody>
</table>

Table 3. The distribution of $^{131}$I-scFv in nude mice at different time points (%ID/g, $\bar{x} \pm s, n=3$)

<table>
<thead>
<tr>
<th></th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>tumor</td>
<td>14.04±1.51</td>
<td>7.78±0.67</td>
<td>5.41±0.92</td>
<td>1.53±0.25</td>
</tr>
<tr>
<td>blood</td>
<td>5.37±0.39</td>
<td>2.74±0.49</td>
<td>1.25±0.53</td>
<td>0.79±0.21</td>
</tr>
<tr>
<td>liver</td>
<td>13.33±1.51</td>
<td>6.07±0.99</td>
<td>1.83±0.06</td>
<td>1.30±0.17</td>
</tr>
<tr>
<td>kidney</td>
<td>9.90±0.36</td>
<td>2.20±0.43</td>
<td>0.87±0.55</td>
<td>0.52±0.13</td>
</tr>
<tr>
<td>cardiac</td>
<td>4.50±0.95</td>
<td>2.58±0.54</td>
<td>0.47±0.09</td>
<td>0.32±0.07</td>
</tr>
<tr>
<td>lung</td>
<td>4.50±1.15</td>
<td>3.22±1.31</td>
<td>0.81±0.06</td>
<td>0.47±0.11</td>
</tr>
<tr>
<td>stomach</td>
<td>3.43±0.64</td>
<td>1.41±0.23</td>
<td>0.37±0.10</td>
<td>0.16±0.06</td>
</tr>
<tr>
<td>intestines</td>
<td>5.13±0.65</td>
<td>3.24±0.87</td>
<td>2.06±0.46</td>
<td>0.72±0.11</td>
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<tr>
<td>brain</td>
<td>2.17±0.70</td>
<td>1.62±0.55</td>
<td>0.71±0.22</td>
<td>0.17±0.03</td>
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<tr>
<td>muscle</td>
<td>2.87±0.45</td>
<td>1.58±0.23</td>
<td>1.15±0.18</td>
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<td>spleen</td>
<td>3.63±0.84</td>
<td>2.25±0.04</td>
<td>0.60±0.41</td>
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Figure 4. Immuno-cytochemistry study in microscope. A. HCC HepG2 positive staining. B. HCC HepG2 negative staining. C. Ovarian cancer SKOV3 negative staining.

However, the properties of some antigens remain unclear, such as the antigens on surface of cancers and antigens on surface of cells differentiated under disease condition. Properties of these antigens are unclear, and even if purified antigens are obtained, antigen conformation remains difficult to simulate the complex environment in vivo due to complexity of the natural conformation of cancer cell antigens and epitope display of the antigens. Because components of the cell surface are complex, it is difficult to obtain antibodies with high affinity if there are more non-specific binding sites, high background value and washing few times during cell screening. While there is loss of specific antibodies if there are washing for more times. Considering the non-specific binding in process of screening, we used subtractive screening in this study [19]. The phage antibody library was screened using normal hepatocytes to exclude the interference of non-specific antibodies. Then, the specific-antibodies were continuously screened using HCC cells. Finally, screening using GPC3 antigens was conducted after 3 rounds of screening to obtain relatively specific antibodies. The experimental results demonstrated that with the increased screening times, the amount of phage becomes...
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Figure 5. SPECT/CT image of tumor-bearing nude mice after 48 h of injection of $^{131}$I-scFv into the tail veins. A. SPECT/CT fusion imaging. B. Three dimensional reconstruction image. C. SPECT imaging.
larger and larger. This gradually improved the recovery rate, and the phage antibodies were enriched significantly. After that, ELISA was performed for the screened phages. The results showed that the positive insertion rate of the antibodies was high, which further confirmed the reliability of the antibody library. ELISA test and western blotting were performed for antibodies with soluble expression, confirming the soluble expression of scFv. According to the literature reports, HepG2 cells and HCC cells exhibit higher expression of GPC3, and the tumorigenicity of HepG2 was good. Therefore, HepG2 and HCC cells are usually used in vitro cytological experiments for HCC [20]. Results of cellular ELISA with specific identification of antibodies suggest that anti-GPC3 single-chain antibodies specifically bind to GPC3 proteins on surface of HepG2 cells. However, anti-GPC3 single-chain antibodies can't bind to SKOV3 cells, indicating that phage antibodies had higher affinity and specificity for HCC cells.

\(^{131}\text{I}\) is widely applied in studies of radio-immunotherapy and radio-immunoimaging because it not only emits \(\beta\)-rays, but also \(\gamma\)-rays. Moreover, the killing effect of \(^{131}\text{I}\) on target cells showed direct ionizing radiation from rays, which does not need internalization of antigen-antibody binding. Therefore, it is considered to be suitable for the treatment of small sub-clinical lesions or lesions with significant clinical symptoms. In addition, it has better immunotherapeutic effect compared to simple antibodies [21].

In this study, anti-GPC3 single-chain antibodies were labeled using \(^{131}\text{I}\) by chloramine-T method. Molecular structure of labeled compounds was stable. These compounds remain stable at room temperature and in serum, showing good stability. \(^{131}\text{I}\)-labeled anti-GPC3 single-chain antibodies were injected into the tumor-bearing nude mice. Images were taken at 4 time points after radioactive drugs were stabilized in mice. \(^{131}\text{I}\)-labeled scFv showed rapid accumulation to tumor sites after injecting into tumor-bearing nude mice. Radioactive materials were mainly accumulated in tumors, gastrointestinal, kidney and liver tissues. Moreover, the radioactivity was gradually decreased with time (%ID/g of kidney and liver decreased from 10% at 12 h to 1%-0.5% after 24 h), suggesting that the radioactive materials were quickly cleared from blood, kidney and liver. Due to specific reaction in tumor regions, the retention time of radioactive materials was long, which was 8.77% at 12 h and 1.53% at 72 h, and were higher than that in other tissues or organs. After 12 h, the ratios of tumor/blood and tumor/muscle for \(^{131}\text{I}\) labeled scFv antibodies were 1.53 and 2.68, respectively, achieving the peaks of 4.7 and 4.8 at 48 h. SPECT imaging also confirmed this radioactive distribution phenomenon. At 48 h, imaging of tumor regions remained clear. Radioactive uptake of other parts of the whole body was lower. Region of interest was plotted to compare the N/T value. The N/T value was the highest at 48 h, suggesting it as the best time point for imaging. Furthermore, \(^{131}\text{I}\) labeled anti-GPC3 single-chain antibodies were specifically enriched to tumor sites in nude mice with good targetability, and can rapidly bind to tumors, predicting that it can be used as a carrier for radio-immunotherapy. Future studies should focus on the anti-tumor effect of \(^{131}\text{I}\)-labeled anti-GPC3 scFv antibody on tumor-bearing nude mice.

Although the present study demonstrated a few interesting results, there are also a few limitations. Firstly, this study is a preliminary investigation exploring the effect of anti-GPC3 single-chain scFv antibody on diagnosis of tumors. The further mechanism for the above effects needs to be clarified in the following studies. Secondarily, the efficacy of anti-GPC3 single-chain scFv antibody also needs to be confirmed and proven through more observational images. Thirdly, the amounts of nude mice involving in this study are relatively less, to confirm the conclusion.

In conclusion, we have successfully constructed a phage antibody library for HCC, and screened anti-GPC3 phage single-chain antibody. Humanized scFv had high affinity and specificity for HCC cells. In vivo experiments showed that \(^{131}\text{I}\)-scFv can specifically accumulate to tumor sites with good targetability, but it required further investigation. Therefore, our study indicated that anti-GPC3 scFv antibody can act as a potential radioactive drug for diagnosing and treating HCC.

Disclosure of conflict of interest

None.

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