Combination of $^{131}$I-anti-endoglin monoclonal antibody and 5-fluorouracil may be a promising combined-modality radioimmunotherapy strategy for the treatment of hepatocellular carcinoma

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ABSTRACT

The present study aimed to investigate the therapeutic efficacy of combined radioimmunoconjugate $^{131}$I endoglin (ENG) and 5-fluorouracil (5-FU) in mouse model with hepatocellular carcinoma (HCC). HCC tumour SMMC7721-GFP xenograft-bearing mice were established and divided into four groups: control group, 5-FU group, $^{131}$I-anti-ENG McAb group, and 5-FU and $^{131}$I-anti-ENG McAb combination group. 5-FU or $^{131}$I-anti-ENG McAb was intraperitoneally injected from Monday to Friday with a therapy break on Saturday and Sunday. So was the combination therapy with 5-FU and $^{131}$I-anti-ENG McAb. $^{131}$I-anti-ENG McAb and $^{131}$I-IgG were prepared, and their biodistribution was studied. Noninvasive fluorescence imaging, tumour volume and tumour weight were measured. High expression levels of ENG in HCC tissues were confirmed by whole-body phosphor-autoradiography. Whole-body phosphor-autoradiography also showed higher tumour uptake for $^{131}$I-anti-ENG McAb compared with $^{131}$I-IgG. Biodistribution studies indicated higher tumour accumulation and better T/NT (6.44 ± 1.01) of $^{131}$I-anti-ENG McAb. Noninvasive fluorescence imaging revealed significant tumour growth suppression in the $^{131}$I-anti-ENG McAb and 5-FU combination therapy group based on reduced fluorescent signals. After treatment with $^{131}$I-anti-ENG McAb and 5-FU, the tumour volume and tumour weight were all decreased. The tumour growth inhibition rate was up to 77.1 ± 4.06% in the $^{131}$I-anti-ENG McAb and 5-FU combination therapy group. The present study demonstrated that the combination of $^{131}$I-anti-ENG McAb and 5-FU may be a promising combined-modality radioimmunotherapy strategy for HCC.

Abbreviations: ENG: endoglin; 5-FU: 5-fluorouracil; TGF-β: transforming growth factor beta; HCC: hepatocellular carcinoma; PBS: phosphate-buffered saline; RIT: radioimmunotherapy; CMRIT: combined modality radioimmunotherapy; GFP: green fluorescent protein; NIFLI: noninvasive fluorescence imaging; VEGF: vascular endothelial growth factor

Introduction

Hepatocellular carcinoma (HCC) is the most common malignant tumour of the liver [1]. Diagnosis and treatment of HCC at an early stage are very important for the prognosis of patients. 5-Fluorouracil (5-FU) is one of the most commonly used chemotherapeutic agents for the treatment of HCC [2]. However, chemoresistance has limited the use of 5-FU alone in clinical regimens [3]. Therefore, combined use of 5-FU and other anticancer agents is considered a therapeutic option for patients with HCC [4].

Antiangiogenic therapy is a highly attractive new method for the treatment of cancers [5]. It potentially overcomes some of the main problems that are related to other therapeutic agents for solid tumours, which result in poor delivery and tumour heterogeneity [6]. Therefore, it is necessary to develop antiangiogenic agents for cancer therapy. Endoglin (ENG), a transmembrane glycoprotein that is mainly overexpressed in activated endothelial cells, is a co-receptor of transforming growth factor beta (TGF-β) that plays an important role in the TGF-β signalling pathway [7,8]. Some studies show that high TGF-β expression correlates with poor prognosis and decreased survival in various solid tumours, including HCC [9,10]. ENG is an ideal marker for angiogenesis because it is almost exclusively expressed in activated endothelial cells [11]. Therefore, ENG has become a potential diagnostic, prognostic and therapeutic target in primary and metastatic HCC.

Radioimmunotherapy (RIT) is a treatment modality for cancer patients. New RIT strategies that increase the
efficacy but limit the toxicity are being investigated [12,13]. Combined modality radioimmunotherapy (CMRIT) is increasingly used in the treatment for solid tumours. It consists of concurrent or sequential use of chemotherapy and external beam radiation. CMRIT has two concepts: radiosensitisation of tumour cells and direct cytotoxic effect of chemotherapy. CMRIT has been utilised in treatments for cancers in the oesophagus, rectum, anus and lungs [14–16]. For these solid tumours, chemotherapy acts as a radiation sensitiser only for local disease. Therefore, CMRIT is most useful for solid tumours in which metastasis is not clinically apparent.

The combined use of antiangiogenic therapy and RIT may be beneficial for the treatment of HCC. Therefore, inhibition of tumour endothelial compartments by antiangiogenic agents and direct action of RIT on tumour cells can be achieved by the combined regimen. Based on this hypothesis, we have examined therapeutic benefits of this combination in a subcutaneous xenograft model of HCC, and revealed the therapeutic efficacy of $^{131}$I-anti-ENG McAb RIT for HCC [17]. In the present study, we assess the impact of the combination of RIT and antiangiogenic therapy on the survival of nude mice injected with the human SMMC7721-GFP cell line. In addition, the effect of low-dose RIT with the chemotherapeutic agent 5-FU is also explored in combined modality RIT.

Materials and methods

Cells

The human HCC cell line SMMC7721-GFP stably expressing green fluorescent protein (GFP) at high levels and the human normal liver cell line L-02 were obtained from the Cell Bank of Shanghai Institute for Biological Sciences (Shanghai, China). The cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 100 U/ml penicillin (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA), and 10% foetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA). The cells in the logarithmic growth phase were used for experiments. After the cells reached confluence, digestion and passage were conducted with 0.25% pancreatin under aseptic conditions. All cells were cultured in a humidified atmosphere with 5% $\text{CO}_2$ at 37 °C. Cells were regularly passaged to maintain exponential growth.

Animals

HCC tumour models were established by subcutaneous injection of $5 \times 10^6$ SMMC7721-GFP cells (200 $\mu$L) into the right upper back of male BAIB/cA-nu mice (20–25 g; 5–8 weeks; Animal Center of Peking University, Beijing, China). The mice were maintained in a protected environment in a laminar flow unit and given sterilised food and water ad libitum. All animal studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee of Shandong University.

Iodogen method

Sodium iodide $[^{131}\text{I}]$ (185 MBq; 50 $\mu$L) was purchased from China Institute of Atomic Energy (Beijing, China). Radioiodination of 240 mg anti-ENG McAb (Santa Cruz Biotechnology, Dallas, TX, USA) and isotype IgG (Biosynthesis Biotechnology Co., Ltd., Beijing, China) with $^{131}$I was performed according to iodogen method as previously described [18]. IgG served as a specific control antibody. The radiolabelling efficiency of $^{131}$I-anti-ENG McAb was 92.4 ± 0.72% and that of $^{131}$I-IgG was 93.6 ± 0.58%. The specific activity of $^{131}$I-anti-ENG McAb was 69.7 ± 6.26 MBq/$\mu$L and that of $^{131}$I-IgG was 71.5 ± 8.24 MBq/$\mu$L.

Chromatography

Radioiodinated anti-ENG McAb and IgG were separated from free iodine using size exclusion columns (PD-10 Sephadex G-25; GE Healthcare, Diegem, Belgium), and the flow-through was collected in sequential fractions. The radiochemical purity was determined by thin-layer chromatography as described with modifications [19]. Thin-layer chromatography was performed using glass plates (Analtech Inc., Newark, DE, USA) containing 6% methanol–chloroform for the evaluation of free acids and other solvents where indicated. For radiochemical analyses, the plates were marked into 10 equal sections and the samples were applied. After development and drying, the plates were cut into sections, and the sections were counted in a $\gamma$-counter. The radiochemical purity of $^{131}$I-anti-ENG McAb was 92.8 ± 0.72% and that of $^{131}$I-IgG was 94.3 ± 0.65%.

Determination of octanol–water partition coefficient

To determine the partition coefficient (log P) of $^{131}$I-anti-ENG McAb, 140–190 kBq (4–5 $\mu$Ci) of the complex was added to a solution containing 1 mL of water and 1 mL of octanol ($n = 3$). The resulting solution was shaken thoroughly for 1 h at room temperature and separated by centrifugation (5 min; 1300 $\times$ g). Counts in aliquots ($n = 3$) of the organic and inorganic layers were determined using a $\gamma$-counter. The partition coefficient was calculated as a ratio of counts in the octanol phase to
counts in the water phase. An average log $P$ value was obtained from these ratios. The partition coefficient log $P$ value for $^{131}$I-anti-ENG McAb was $2.58 \pm 0.33$.

**Whole-body autoradiography**

Dynamic whole-body phosphor-autoradiography was performed using the Cyclone Plus Storage Phosphor System (PerkinElmer, Waltham, MA, USA). Potassium iodide (1%) was added to drinking water two days before injection of $^{131}$I-labeled antibody to block the thyroid gland. Six days after injection of SMMC7721-GFP cells, $^{131}$I-anti-ENG McAb or $^{131}$I-IgG (200 $\mu$L, 0.37 MBq) was injected into the mice through the tail vein. Whole-body autoradiography was performed at 1, 12, 24, 48 and 72 h after injection. The mice were anesthetised by intraperitoneal injection of 0.60% pentobarbital sodium (0.1 mL/10 g body weight). Anesthetised mice were fixed on the storage phosphor screen plate in supine position with four limbs stretched in order to make the tumour tightly close to the plate. The plate was exposed to a mouse for 15 min in a darkroom. After exposure, the plate was scanned by Cyclone Plus Storage Phosphor System and analysed using OptiQuant Acquisition software (PerkinElmer, Waltham, MA, USA).

**Detection of biodistribution of $^{131}$I-anti-ENG McAb and $^{131}$I-IgG**

For the biodistribution study, HCC tumour model mice were injected with $^{131}$I-anti-ENG McAb or $^{131}$I-IgG (200 $\mu$L, 0.37 MBq). Six mice were euthanized at 1 h, 12 h, 24 h, 48 and 72 h after injection in each group. Blood, tumour, muscular tissue on the left side and main organs were excised, rinsed to remove residual blood and weighed. Samples and primed standards were evaluated for radioactivity in the $\gamma$-counter and were corrected for physical decay. Tissue activity is expressed as the percent injected dose per gram (%ID/g). The target-to-non-target ratio was defined as tumour to muscle (T/NT).

**Noninvasive fluorescence imaging (NIFLI)**

When the tumour volumes reached an average of approximately 50 mm$^3$, the mice were randomly assigned to one of the following treatment groups ($n = 6$ for each group): i) untreated group (phosphate-buffered saline (PBS); control group), ii) 5-FU treatment group (8 mg/kg) that was intraperitoneally injected from Monday to Friday with a therapy break on Saturdays and Sundays [20], iii) $^{131}$I-anti-ENG McAb group (McAb dose was chosen based on a dose-dependent titration experiment; the dose was 0.5 $\mu$g/g), and iv) the combination group that received therapy with both 5-FU and $^{131}$I-anti-ENG McAb (5-FU and $^{131}$I-anti-ENG McAb were used simultaneously as described above) [21]. The tumour volumes (mm$^3$) were calculated using the following formula: length $\times$ width$^2 \times 0.5$. The relative tumour proliferation rate was calculated as $V_{\text{Treatment}} / V_{\text{Control}}$ where $V_{\text{Treatment}}$ is the tumour volume of either treatment group and $V_{\text{Control}}$ is the tumour volume of the untreated group. All of the mice were treated for 3 weeks. The whole experiment lasted for one month. At the end of the experiment, tumour tissues from each animal were excised and then weighed. The tumour weight evaluation formula was $I$ ($\%$) = $[(M_t - M_c)/M_c] \times 100\%$, where $I$ is the tumour growth inhibition rate, $M_t$ is the average tumour weight in either treatment group and $M_c$ is the average tumour weight in the untreated group. In vivo GFP images were obtained using the in vivo FX Pro Imaging System (Kodak, Rochester, NY, USA) that used appropriate filters (excitation at 445–490 nm, emission at 535 nm). Activity was quantified by viewing the region of interest in the tumour analysis software molecular imaging standard edition (Carestream, Shanghai, China). Signal intensities from manually derived regions of interest were expressed as photon flux (counts/sec). The tumor growth was presented as photon flux $\times 10^2$ $\times$ mm$^{-2}$ $\times$ sec$^{-1}$. Fluorescent signals were presented in colour: blue for the lowest intensity and red for the highest intensity.

**Statistical analysis**

SPSS 18.0 statistical software package (IBM, Armonk, NY, USA) was used to analyse the results. The data were expressed as means with standard deviations ($\pm$SD). One-way analysis of variance (ANOVA) was used for group comparison. Differences between experimental results were considered statistically significant if $P < 0.05$.

**Results and discussion**

**ENG is widely expressed in HCC tumour tissues, and $^{131}$I-anti-ENG McAb specifically targets HCC**

To identify the location of ENG, whole-body autoradiography was carried out. HCC tumours in the $^{131}$I-anti-ENG McAb group were more clearly visible at 12, 24 and 48 h with high contrast to the contralateral background than those in the $^{131}$I-IgG group and the accumulation reached a peak at 24 h. The images also showed that there was non-specific accumulation in $^{131}$I-IgG group (Figure 1). The result suggests that ENG is widely expressed in HCC tumour tissues, and $^{131}$I-anti-ENG McAb specifically targets HCC.
HCC tumour has high expression of ENG, and $^{131}$I-anti-ENG McAb can detect the expression of ENG at an early time

To validate the imaging studies and further quantify the $^{131}$I-anti-ENG McAb uptake, we detected the biodistribution of $^{131}$I-anti-ENG McAb and $^{131}$I-IgG at 1, 12, 24, 48 and 72 h (Table 1). The biodistribution study revealed that blood pool activity was prominent at 24 h, and other tissues with noticeable uptake were kidneys and spleen. All remaining tissues had a low %ID/g, being in agreement with the imaging data (Figure 2(A)). In the $^{131}$I-anti-ENG McAb group, T/NT reached a peak at 24 h after injection (T/NT ratio = 6.44 ± 1.01). However, T/NT remained comparatively stable throughout the experiment in the $^{131}$I-IgG group (Figure 2(B)). The tumour uptake was 4.58 ± 0.01, 3.56 ± 0.02, 3.76 ± 0.12, 1.69 ± 0.02 and 0.52 ± 0.01%ID/g at 1, 12, 24, 48 and 72 h, respectively, for $^{131}$I-anti-ENG McAb, and 1.58 ± 0.03, 0.86 ± 0.02, 0.61 ± 0.12, 0.37 ± 0.019 and 0.052 ± 0.01%ID/g at 1, 12, 24, 48 and 72 h, respectively, for $^{131}$I-IgG. Comparable kidney, spleen and liver activities were observed for radiotracers, and the kidney uptake decreased from 6.90 ± 0.05%ID/g at 1 h to 0.35 ± 0.04%ID/g at 72 h after injection of $^{131}$I-anti-ENG McAb. Meanwhile, the spleen uptake decreased from 5.73 ± 0.04%ID/g at 1 h to 0.15 ± 0.05%ID/g at 72 h after injection of $^{131}$I-anti-ENG McAb (Figure 2(C)). These results indicate that HCC tumour has high expression of ENG, and $^{131}$I-anti-ENG McAb can detect the expression of ENG at an early time.

Tumour growth and proliferation in $^{131}$I-anti-ENG McAb and 5-FU combination therapy group are significantly slower than in all other groups

To explore the therapeutic effects of the combination therapy of both 5-FU and $^{131}$I-anti-ENG McAb in vivo, we established a mouse xenograft model using SMMC7721-GFP cells. The data showed that continuously increasing fluorescent signals existed in untreated mice, whereas a reduction in tumour burden was observed in mice treated with 5-FU, $^{131}$I-anti-ENG McAb and the combination of $^{131}$I-anti-ENG McAb and 5-FU (Figure 3(A)). The tumour growth in the untreated group was higher than that in the other groups (Figure 3(B)). The tumour growth in the combination therapy group was significantly slower than in all other groups.

Table 1. Biodistribution of $^{131}$I-Anti-ENG mAb in HCC mice.

| Tissues or organs | 1       | 12      | 24      | 48      | 72      |
|-------------------|---------|---------|---------|---------|---------|
| Blood             | 6.470 ± 0.0685 | 4.672 ± 0.0978 | 2.510 ± 0.161 | 1.622 ± 0.0524 | 0.294 ± 0.0467 |
| Liver             | 6.140 ± 0.0857 | 4.250 ± 0.0646 | 2.13 ± 0.1534 | 2.078 ± 0.0483 | 0.151 ± 0.0482 |
| Kidney            | 6.904 ± 0.0526 | 4.819 ± 0.0686 | 3.200 ± 0.1462 | 2.638 ± 0.0407 | 0.351 ± 0.0394 |
| Spleen            | 5.727 ± 0.042 | 3.001 ± 0.021 | 2.762 ± 0.131 | 2.039 ± 0.0294 | 0.109 ± 0.0372 |
| Stomach           | 2.965 ± 0.016 | 1.384 ± 0.0207 | 1.007 ± 0.126 | 0.284 ± 0.0153 | 0.0173 ± 0.0018 |
| Intestine         | 2.861 ± 0.030 | 1.511 ± 0.0304 | 1.021 ± 0.124 | 0.286 ± 0.0241 | 0.0196 ± 0.0021 |
| Heart             | 1.570 ± 0.052 | 0.967 ± 0.0587 | 0.430 ± 0.146 | 0.255 ± 0.0431 | 0.0467 ± 0.0033 |
| Lung              | 5.766 ± 0.042 | 3.118 ± 0.0531 | 1.110 ± 0.1391 | 0.206 ± 0.0352 | 0.139 ± 0.0263 |
| Bone              | 1.806 ± 0.0162 | 0.917 ± 0.0292 | 0.53 ± 0.1156 | 0.205 ± 0.0126 | 0.125 ± 0.0164 |
| Thyroid           | 3.754 ± 0.0357 | 3.179 ± 0.0450 | 0.876 ± 0.136 | 0.710 ± 0.0294 | 0.136 ± 0.0351 |
| Muscle            | 1.680 ± 0.0316 | 0.810 ± 0.0495 | 0.520 ± 0.121 | 0.360 ± 0.0163 | 0.095 ± 0.0124 |
| Tumor             | 4.584 ± 0.013 | 3.560 ± 0.021 | 3.764 ± 0.116 | 1.487 ± 0.017 | 0.332 ± 0.0120 |

Note: Data are presented as means %ID/g ± standard deviations (n = 5).
significantly slower than that in the 5-FU group or the $^{131}$I-anti-ENG McAb group ($P < 0.05$) (Figure 3(C)). At the end of therapy, the animals were killed, and tumour blocks were dissected and weighed. Figure 4(A) shows four representative tumours in each group. The relative tumour proliferation rate ($V_{\text{Treatment}} / V_{\text{Control}}$) in $^{131}$I-anti-ENG McAb and 5-FU combination therapy group was slower than that in any other group ($P < 0.01$) (Figure 4(B)). Similarly, the tumour blocks in the $^{131}$I-anti-ENG McAb and 5-FU combination therapy group weighed less than those in any other group (Figure 4(C)). Consistently, the tumour growth inhibition rate in $^{131}$I-anti-ENG McAb and 5-FU combination therapy was greater than that of any other group (Figure 4(D)). These results suggest that the tumour growth and proliferation in $^{131}$I-anti-ENG McAb and 5-FU combination therapy group were significantly slower than in all other groups.

Comparative analysis

Angiogenesis is important at the early stage of multistep hepatocarcinogenesis [22]. HCC typically develops from dysplastic nodules in a cirrhotic liver. Endothelial cells in dysplastic nodules usually have phenotypic changes in malignant transformation, which is demonstrated by changes in endothelial cell markers, providing rational targets for innovative HCC therapy. Anti-angiogenic therapy breaks the tumour vasculature, and deprives the tumour of oxygen and nutrients. Bevacizumab, also named Avastin, is an antiangiogenic drug that binds vascular endothelial growth factor (VEGF) [23,24]. The combination of bevacizumab with gemcitabine and oxaliplatin has been tested in 33 patients with unresectable HCC. Of these patients, 18% had partial responses and 24% had disease stabilisation [25]. However, other angiogenic pathways are activated after VEGF-mediated
signalling is blocked by bevacizumab, resulting in drug resistance.

ENG regulates TGF-β receptor complex that tightly binds TGF-β1 and -β3 [7,8]. Overexpression of ENG modulates the cellular responses to TGF-β1, including inhibition of cellular proliferation and down-regulation of c-myc mRNA, stimulation of fibronectin synthesis, cellular adhesion, platelet-endothelial cell adhesion molecule-1 phosphorylation, and homotypic aggregation. ENG is an ideal marker in the quantification of intra-tumoural microvessel density in different solid tumours such as breast, prostate, cervical, colorectal and HCC in addition to CD34 and CD31, which are usually used as endothelial cell markers [26,27]. Overexpression of hepatic ENG is discovered in HCC tissues and correlated with carcinogenesis, progression and prognosis of HCC [28–30]. These studies suggest that assessment of neovascularization by ENG staining during carcinogenesis might be useful in the prediction of cancer development.

Autoradiography imaging in the present study shows that 131I-anti-ENG McAb accumulation at tumour site

Figure 4. Tumour prevention study. (A) Representative tumour images from different groups on day 31. (B) Relative tumour proliferation rate, \( V_{\text{Treatment}}/V_{\text{Control}} \). *, \( P < 0.05 \) and **, \( P < 0.01 \). (C) Tumour weights of the three groups on day 31. (D) Tumour growth inhibition rates.
becomes visible from 12 h after injection, and is increased continually. These results reveal that HCC tissues can specifically uptake $^{131}$I-anti-ENG McAb. In addition, biodistribution data show that $^{131}$I-anti-ENG McAb has a high tumour uptake and T/NT ratio, and demonstrate the high expression of ENG in vivo and in vitro, as well as the specific binding of $^{131}$I-anti-ENG McAb with ENG in HCC. These findings provide supportive evidence for the usefulness of ENG as a specific target in imaging and antiangiogenic therapy of HCC at an early stage.

The combination of anti-angiogenic therapy with other therapies such as chemotherapy and radiotherapy in tumour treatment may enhance the therapeutic efficacy compared with either therapy alone [31]. Because of its cell-killing activity, 5-FU has become the most commonly used chemotherapeutic agent against HCC. Chemoresistance has precluded the clinical use of 5-FU alone. A variety of combination chemotherapy regimens have been studied in HCC [32]. In the present study, the relative rate of tumour proliferation and the weight of tumour blocks in the $^{131}$I-anti-ENG McAb and 5-FU combination therapy group were lower and lighter than that in the other groups. In addition, the combination of $^{131}$I-anti-ENG McAb and 5-FU had anti-tumour efficacy and completely suppressed the established tumours in the HCC xenografts. The study showed that the combination of $^{131}$I-anti-ENG McAb with 5-FU caused regression of HCC xenografts in nude mice.

This study, however, has some limitations. First, the sample size was relatively small. Second, the results were preliminary and the underlying mechanisms were unclear. Future studies with a larger sample size to reveal the underlying mechanism are needed.

Conclusions

The present study demonstrated that $^{131}$I-anti-ENG McAb and 5-FU are more effective in reducing the tumour size of HCC in a mouse model than $^{131}$I-anti-ENG McAb or 5-FU treatment alone. This suggests that $^{131}$I-anti-ENG McAb and 5-FU may be used as an effective new modality for the treatment of patients with HCC. To uncover the underlying mechanism of action, further studies with a larger sample size are needed.

Disclosures statement

All authors declare no financial competing interests.

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