**ORIGINAL ARTICLE**

I\(^{131}\) reinforces antitumor activity of metuximab by reversing epithelial–mesenchymal transition via VEGFR-2 signaling in hepatocellular carcinoma

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**INTRODUCTION**

Hepatocellular carcinoma (HCC) is a common malignant tumor that is commonly treated with surgery, minimally invasive treatment and transcatheter arterial chemoembolization (TACE) (Li & Yeo, 2017; Ramaswami et al., 2016). However, postoperative recurrence and metastasis remain important factors that affect the survival of patients with HCC (Grandhi et al., 2016; Yagci, Cetin, & Ercin, 2017). In particular, in HCC treated with chemotherapy or TACE, the
Immunotherapy is an important approach of comprehensive cancer therapy. Specifically, HCC cells strongly express CD147 antigen, which is related to the invasion and metastasis of HCC (Chen et al., 2016). Thus, CD147, a highly glycosylated transmembrane protein and a member of the immunoglobulin superfamily, is an effective target for HCC immunotherapy. CD147 expression is up-regulated in many tumors and is especially high in HCC tissue and HCC cell lines, where it is expressed in up to 75%. Conversely, it is not expressed in normal hepatic tissue and normal hepatic cell lines (Dai et al., 2009; Gou et al., 2009). Moreover, the over-expression of CD147 can induce the expression and secretion of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), which degrade extracellular matrix (ECM) and promote tumor invasion and metastasis by interfering with mesenchymal cells (Cui et al., 2012; Wang et al., 2010). To target CD147, a highly specific monoclonal antibody, HAB18 or metuximab, was developed and conjugated to the radioisotope $^{131}$I. The resultant $^{131}$I-metuximab ($^{131}$I-mab) is a radio-immunotherapy injection that was officially approved by the CFDA in 2007, being under the trade name Licartin for the treatment of local HCC (Ma & Wang, 2015; Wu, Shen, Xia, & Yang, 2016). $^{131}$I-mab exhibits several advantages, such as its specificity, rapid response and lethality to cancer cells. The preliminary results of a phase IV clinical trial of TACE combined with $^{131}$I-mab for the treatment of advanced HCC at eight Chinese institutions showed that $^{131}$I-mab is a safe and effective agent for the treatment of advanced HCC (Xu et al., 2007) and can effectively delay HCC relapse after hepatic transplantation (Wu et al., 2010, 2012). Moreover, the combination of TACE and $^{131}$I-mab can significantly prolong progression-free survival and the overall survival of patients with advanced HCC according to the Barcelona clinical staging (BCLC) (Li et al., 2009). Among the 167 patients with HCC in this study, the 1-year survival rate was significantly higher for patients receiving the combination treatment than that of patients only receiving the TACE treatment. The extrahepatic metastasis incidence in the combined treatment group was 2.94%, which was significantly lower than the 8.57% rate in the TACE group. This difference suggests that $^{131}$I-mab can reduce the extrahepatic metastasis incidence in patients after TACE treatment.

2 | RESULTS

2.1 | Inhibition of HCC cell proliferation by $^{131}$I-mab

Western blotting was used to measure the expression levels of CD147 in a variety of cell lines, and CD147 was expressed in HepG2, Hep3B, SMMC-7721, MHCC97H and MHCC97L cells, with HepG2, Hep3B and MHCC97H cells expressing higher levels of CD147. MHCC97L cells expressed the lowest level of CD147, and WRL-68 cells were
negative for the expression of CD147 (Figure 1a). Based on these results, we selected CD147-negative WRL-68 cells to investigate the cytotoxicity of I\textsuperscript{131}-mab and CD147 antibody (CD147-mab). CD147-mab did not affect cell viability, whereas I\textsuperscript{131}-mab exerted a dose-dependent effect on cell viability. The IC\textsubscript{20} and IC\textsubscript{50} values of I\textsuperscript{131}-mab on WRL-68 cells were 8.09 μCi/100 μl and 12.69 μCi/100 μl, respectively (Figure 1b). Because WRL-68 cells did not express CD147 and were not inhibited by CD147-mab, the cytotoxicity of I\textsuperscript{131}-mab was attributed to the conjugated I\textsuperscript{131}. Because the unit of measurement of I\textsuperscript{131}-mab and I\textsuperscript{131} was μCi and the unit of measurement of CD147-mab is μM, therefore, to establish the concentration equivalence relation between I\textsuperscript{131}-mab and CD147-mab, we regarded that the concentrations of I\textsuperscript{131}-mab corresponding to IC\textsubscript{20} and IC\textsubscript{50} could be served as the reference concentrations of I\textsuperscript{131} and consequently calculated the molar concentrations of CD147-mab to be 5.62 μM/100 μl and 8.82 μM/100 μl, respectively.

The tested cells were treated with I\textsuperscript{131}-mab, I\textsuperscript{131} (both agents administered at doses of 8.09 μCi/100 μl or 12.69 μCi/100 μl) and CD147-mab (5.62 μM/100 μl and 8.82 μM/100 μl) to examine their...
effects on the proliferation of HCC cells. The results showed that $^{131}$I-mab was significantly more cytotoxic to all HCC cell lines than CD147-mab or $^{131}$I alone, and specifically, viability was lowest in HepG2, Hep3B and MHCC97H cells, and doses of 8.09 μCi/100 μl and 12.69 μCi/100 μl resulted in viabilities lower than 30% and 10%, respectively. In contrast, $^{131}$I-mab was weakly cytotoxic to MHCC97L, resulting in viabilities exceeding 50% at doses of 8.09 μCi/100 μl and 12.69 μCi/100 μl. Moreover, CD147-mab was significantly more cytotoxic to HepG2, Hep3B, SMMC-7721 and MHCC97H cells than $^{131}$I, whereas the effects of $^{131}$I and CD147-mab on MHCC97L cells did not significantly differ (Figure 1c).

2.2 Inhibition of HCC cell invasion by $^{131}$I-mab

To verify the effect of $^{131}$I-mab on HCC cell invasion, a Transwell device was used to assess cell invasion. Both $^{131}$I-mab and CD147-mab significantly inhibited the invasion of the HCC cell lines. Specifically, $^{131}$I-mab inhibited HepG2, Hep3B, SMMC-7721, MHCC97H and MHCC97L cell invasion more effectively than CD147-mab, whereas the effects of CD147-mab on the invasion of Hep3B and MHCC97L did not significantly differ from the control group. These results suggested that inhibition of HCC cells by $^{131}$I-mab or CD147-mab was closely associated with CD147 expression. $^{131}$I alone exerted a limited effect on HCC cell invasion, and this effect only differed between the treated and control groups for MHCC97L cells (Figure 2).

2.3 $^{131}$I-mab reversed HCC cell EMT via VEGFR-2 signaling

To elucidate the mechanism by which $^{131}$I-mab inhibits HCC cells, we compared some markers related to EMT in HCC cells treated with $^{131}$I-mab, $^{131}$I and CD147-mab. E-cadherin expression was significantly increased, whereas

**FIGURE 2** Inhibition of HCC invasion by $^{131}$I-mab. The upper Transwell chambers were coated with 50 μl of 1:6 diluted Matrigel (BD Biosciences, San Jose, USA), and the lower chambers were filled with 500 μl of 10% fetal bovine serum, and the Transwell chambers were placed in a well of a 24-well plate. HCC cells were seeded into the upper chamber of the Transwell (1 × 10^5 cells/well) and cultured for 24 hr; the cells were treated with $^{131}$I-mab, $^{131}$I (8.09 μCi/100 μl) and CD147-mab (5.62 μM/100 μl) were used to treat cells for 48 hr. The Transwell chambers were collected, and the cells were stained with 0.1% crystal violet for 20 min. Positive cells were counted. Compared with the control group:

*p < .05, **p < .01 and ***p < .001
the expression levels of N-cadherin and vimentin were decreased in all HCC cells treated with I\textsuperscript{131}-mab and CD147-mab. Conversely, I\textsuperscript{131} did not affect the expression levels of these proteins (Figure 3a). These results suggested that I\textsuperscript{131} mab reversed HCC cell EMT via the CD147 antibody in the conjugated molecules but not via I\textsuperscript{131}.

VEGFR expression was further studied in HCC cells treated with I\textsuperscript{131}-mab, CD147-mab and I\textsuperscript{131}. The results showed that VEGFR-2 phosphorylation (p-VEGFR-2) significantly decreased in HCC cells treated with I\textsuperscript{131}-mab and CD147-mab, whereas the levels of VEGFR-1, p-VEGFR-1 and VEGFR-2 did not change. I\textsuperscript{131} alone did not affect the expression levels of these proteins in HCC cells (Figure 3b). These results suggest that I\textsuperscript{131}-mab reversed EMT by inhibiting the phosphorylation of VEGFR-2 in HCC cells.

2.4 | I\textsuperscript{131} increases the sensitivity of HCC cells to the cytotoxicity of CD147-mab

By MTT assay, we found that I\textsuperscript{131} did not inhibit the proliferation of all HCC cells at doses <8.09 μCi/100 μl, whereas the cytotoxicity of 5.62 μM/100 μl CD147-mab (concentration corresponding to the I\textsuperscript{131} dose) varied among HCC cell lines. We cotreated cells with I\textsuperscript{131} (8.09 μCi/100 μl) and CD147-mab (5.62 μM/100 μl) to compared the effect of this treatment to that of CD147-mab alone. We found that the viability of all HCC cell lines significantly decreased in response to the combined treatment (Figure 4a). The results of cell invasion capability were coincident with the MTT assay (Figure 4b,c). The results suggested that I\textsuperscript{131} increases the sensitivity of HCC cells to CD147-mab and consequently enhances the cytotoxicity of this agent.

2.5 | Inhibition of SMMC-7721 human hepatoma xenografts by I\textsuperscript{131}-mab

The inhibition of HCC by I\textsuperscript{131}-mab and the molecular mechanisms underlying this inhibition were verified in nude mice harboring SMMC-7721 human hepatoma xenografts. Treating these xenografts with I\textsuperscript{131}-mab injection markedly inhibited tumor growth speed; specifically, the tumor volume was significantly smaller in the I\textsuperscript{131}-mab treatment group than that in the control group 7 days after the first injection, and this difference increased over time. The efficacy of CD147-mab group was evident 14 days after the start of treatment, whereas no noticeable effects were observed in the I\textsuperscript{131} treatment group, even at the end of the observation period (Figure 5a). After 21 days of treatment, the tumor volume in the control group exceeded the standard limit, and the observation was ended. The tumors were dissected and weighed, and the tumor weight was lowest in the I\textsuperscript{131}-mab group, followed by the CD147-mab group. There is no difference in tumor weight between the I\textsuperscript{131} group and the control group (Figure 5b). Tissue sections were obtained from the hepatoma xenografts, and immunohistochemistry staining was used to measure E-cadherin and p-VEGFR-2 expression. The results showed that E-cadherin expression was up-regulated, whereas p-VEGFR-2 expression was down-regulated in the I\textsuperscript{131}-mab and CD147-mab groups (Figure 5c).

3 | DISCUSSION

EMT has been observed in many human tumors, and extracellular signaling molecules that induce EMT in tumor cells include MMP-2, MMP-3, MMP-9, type I or III collagen, hepatocyte growth factor (HGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), TGF-β and tumor necrosis factor-α (TNF-α). EMT also modulates the activity of multiple signaling pathways, such as TGF-β, Wnt, PDGF, Notch, Hedgehog, Akt, PI3K, NF-κB and Ras, via the Snail family of zinc finger transcription factors (Snail1, Snail2 and Snail3) and the helix-loop-helix structure of transcription factors (Twist, ZEB 1 and ZEB2/SIP1) to down-regulate the epithelial markers E-cadherin and keratin or up-regulate the mesenchymal markers N-cadherin and vimentin (Hanna & Shevde, 2016; Lee & Kong, 2016; Moustakas & Heldin, 2008; Lee & Kong, 2016; Moustakas & Heldin, 2008). EMT increases the malignancy of cancer cells because cells lose apical–basal polarity and cell junctions and acquire a migratory mesenchymal phenotype. These cells then invade the lymphatic or vascular system and spread to different sites or organs to grow and form metastatic tumors (Jayachandran et al., 2016; Köbl, Jeschke, & Andergassen, 2016).

TACE is an important treatment for HCC but has been clinically associated with increases in distant metastasis due to residual cancer cells in many patients with HCC (Xue et al., 2016). This phenomenon has greatly compromised the long-term efficacy of TACE. Moreover, TACE can induce tissue hypoxia, which down-regulates E-cadherin and up-regulates of N-cadherin in residual cancer cells. Thus, hypoxia may mediate the negative effects of TACE by inducing EMT in cancer cells (Fransvea, Angelotti, Antonaci, & Giannelli, 2008; Xue et al., 2015). EMT enhances the invasion and metastasis of cancer cells, reduces the sensitivity or increases the resistance of HCC to chemotherapeutic agents (Bae et al., 2014; Nishida, Angelotti, Antonaci, & Giannelli, 2008; Xue et al., 2015). EMT enhances the invasion and metastasis of cancer cells, reduces the sensitivity or increases the resistance of HCC to chemotherapeutic agents (Bae et al., 2014; Nishida, Angelotti, Antonaci, & Giannelli, 2008; Xue et al., 2015). EMT enhances the invasion and metastasis of cancer cells, reduces the sensitivity or increases the resistance of HCC to chemotherapeutic agents (Bae et al., 2014; Nishida, Angelotti, Antonaci, & Giannelli, 2008; Xue et al., 2015).
FIGURE 3  Impact of $^{131}$I-mab on EMT and VEGFR-2 expression in HCC cells. (a) HCC cells were seeded in 24-well plates ($1 \times 10^5$ cells/100 μl/well) and cultured for 24 hr. $^{131}$I-mab and CD147-mab were diluted in serum-free medium. The cells were then treated with $^{131}$I-mab, $^{131}$I (8.09 μCi/100 μl) or CD147-mab (5.62 μM/100 μl) for 48 hr. The cells were harvested, total protein was extracted, and the protein concentration was measured. Western blotting was used to measure the expression of E-cadherin, N-cadherin and vimentin. GAPDH was used as a loading control. Compared with the parental cell group: *$p < .05$, **$p < .01$ and ***$p < .001$. (b) Cells were cultured and treated as described above. Western blotting was used to measure the expression of VEGFR-1, p-VEGFR-1, VEGFR-2 and p-VEGFR-2. GAPDH was used as a loading control. Compared with the parental cell group: *$p < .01$ and ***$p < .001$
HCC cells (Baldassarre et al., 2012). Blocking the effects of upstream loop regulatory factors of EMT, such as inhibition of NF-κB/miR-448, can improve the response of cancer cells to chemotherapeutic agents (Li et al., 2011). CD147, under the regulation of slug at transcriptional level, can promote EMT process in HCC via TGF-β signaling (Wu et al., 2011). Furthermore, hypoxia caused by TACE can up-regulate CD147 expression in HCC cells (Gou et al., 2016), and the over-expression of CD147 enhances EMT in cancer cells by activating TGF-β signaling. Thus, ${}^{131}$I-mab, which targets CD147, may specifically inhibit EMT to attenuate tumor development and metastasis. In nude mouse HCC models, ${}^{131}$I-mab effectively inhibited the growth and metastasis of HCC and inhibited MMPs and VEGF expression in the para-tumor microenvironment (Wu et al., 2011). We conducted a prospective controlled clinical study in which the combination of TACE and ${}^{131}$I-mab was used to treat intermediate-stage HCC (BCLC staging). Specifically, 68 patients with intermediate-stage HCC were included in the combined treatment group, and 70 patients were included in the TACE alone group. The median survival time was 26.7 months in the combined treatment group but only 20.6 months in the control group, and the overall survival in the combined treatment group was significantly better than that in the control group ($p = .038$). The median time to progression was 18.6 months in the combined treatment group and superior to only 12.5 months in the control group ($p = .046$). In particular, the extrahepatic metastasis rate was 2.94% in the combined treatment group and 8.57% in the TACE group. These results suggested that ${}^{131}$I-mab reduces the risk of extrahepatic metastasis after TACE treatment (Wu et al., 2012).

In this work, the cytological experiments proved that ${}^{131}$I-mab inhibits the proliferation and invasion of HCC cells, and this inhibition is closely related to the level of CD147 expression. HCC cells expressing high levels of CD147 are more sensitive to ${}^{131}$I-mab. The inhibition effect of ${}^{131}$I-mab on HCC cells was mostly attributed to the CD147 antibody within the conjugated molecule. Although the inhibition of HCC cell proliferation and invasion by ${}^{131}$I alone is limited, when combined with CD147-mab, ${}^{131}$I significantly enhanced the sensitivity of cancer cells to CD147-mab and consequently enhanced the cytotoxicity of anticancer antibodies. This finding suggested that the two components of ${}^{131}$I-mab synergistically inhibited HCC. Both ${}^{131}$I-mab and CD147-mab can reverse the EMT of HCC cells partially by inhibiting the phosphorylation of VEGFR-2 and then reduce the capabilities of proliferation and metastasis of HCC cells. The experiments using SMMC-7721 human hepatoma xenografts in athymic nude mice also proved that ${}^{131}$I-mab and CD147-mab

![Image](https://example.com/image.png)
significantly inhibited xenograft tumors, and $^{131}$I-mab was more effective than CD147-mab in this inhibition. The synergistic effect of conjugated $^{131}$I-mab was attributed to $^{131}$I because $^{131}$I alone did not significantly inhibit the proliferation and invasion of HCC. Our results elucidated the mechanism underlying the antiproliferative and antimetastatic effects of $^{131}$I-mab and provided a theoretical foundation for the clinical application of $^{131}$I-mab.

4 | EXPERIMENTAL PROCEDURES

4.1 | Cells and reagents

Human hepatoma cell lines (HepG2, Hep3B, SMMC-7721, MHCC97H and MHCC97L) and normal hepatic cells (WRL-68) were provided by Cell Bank, Institute of Cell Biochemistry, Chinese Academy of Sciences Shanghai.
Institutes for Biological Sciences (Shanghai, China). The cells were cultured according to the manufacturer’s protocol. I\(^{131}\)-mab was developed by the Chengdu Hua Sun Group Inc., Ltd. (Chengdu, China). CD147-mab and Sodium Iodide [131] Capsules (I\(^{131}\)) were purchased from Abcam Trading Company Ltd. (Shanghai, China) and Atom-Hitech Co., Ltd. (Beijing, China), respectively.

4.2 | Cell proliferation assay

A tetrazolium colorimetric assay (MTT assay) was used to assess the effects of I\(^{131}\)-mab, CD147-mab and I\(^{131}\) on the proliferation of HCC and normal cells. Briefly, cells were harvested during the logarithmic growth phase and seeded in 96-well plates at 1 × 10\(^4\) cells/100 μl/well, and then cultured for 24 hr; serum-free culture medium was used to dilute I\(^{131}\)-mab, and cells were treated with various concentrations of this agent, with eight duplicates per concentration. After incubated for 2 hr, the medium was then replaced with medium containing 10% serum (100 μl/well). After 48 hr of culture, the medium was replaced with 0.1 M PBS solution (100 μl/well), and MTT labeling reagent (10 μl/well for a culture, the medium was replaced with 0.1 M PBS solution containing 10% serum (100 μl/well). After 48 hr of incubation, the absorbance was measured at 490 nm, and the values were plotted to assess the solubilization solution (10% SDS in 0.01 mol/L HCl, Shanghai, China) was then added. After 4 hr of incubation, the final concentration of 0.5 mg/ml; Roche Diagnostics GmbH, Germany) was then added. After 4 hr of incubation, the absorbance was measured at 490 nm, and the values were plotted to assess the cell viability and calculate the IC\(_{50}\) and IC\(_{50}\) of each concentration of I\(^{131}\)-mab and its controls.

4.3 | Cell invasion assay

The upper Transwell chambers (8 μm pore size, Corning, Tewksbury, USA) were coated with 50 μl of 1:6 diluted Matrigel (BD Biosciences, San Jose, USA), and the lower chambers were filled with 500 μl of 10% fetal bovine serum. The Transwell chambers were placed in a well of a 24-well plate. Harvested cells were seeded into the upper Transwell chamber at 1 × 10\(^5\) cells/well and cultured for 24 hr; I\(^{131}\)-mab was diluted in serum-free culture medium and added to the wells for a variety of final concentrations. The cells were incubated for 2 hr, and the medium was replaced with medium containing 10% serum (100 μl/well) after 48 hr of incubation. The Transwell inserts were removed, and the cells were stained with 0.1% crystal violet for 20 min before being counted under a microscope; three random fields were photographed (200× magnification).

4.4 | Immunoblotting

The above-studied cells were seeded in a 24-well plate at 1 × 10\(^5\) cells/well and cultured for 24 hr. I\(^{131}\)-mab was diluted in serum-free culture medium and added to the culture wells at various concentrations (based on the protocol). These cells were cultured for 2 hr. Medium containing 10% serum (100 μl/well) was used to replace the previous medium. After 48 hr of incubation, the cells were harvested. Western blotting was used to measure the expression of the target proteins. The antibodies used in the experiments are listed below: rabbit anti-CD147 (Abcam Trading Company Ltd., Shanghai, China); mouse anti-E-cadherin, mouse anti-N-cadherin, mouse antivimentin, rabbit anti-VEGF-C antibodies (Cell Signaling Technology, Danvers, MA, USA); and mouse anti-VEGFR-1, mouse anti-VEGFR-2, rabbit anti-phospho-VEGFR-1 and rabbit anti-p-VEGFR-2 (Cell Applications Inc., CA, USA).

4.5 | Human hepatoma xenografts in nude mice

A total of 25 healthy, purebred BALB/C male mice aged 4 weeks were purchased from the SLAC Experimental Animal Centre of the Chinese Academy of Sciences (Shanghai, China). SMMC-7721 cells were harvested during the logarithmic growth phase, and cell suspension was prepared. The cells (5 × 10\(^5\) cells in 100 μl per mouse) were subcutaneously injected into the right flanks of nude mice, and tumors formed 12 days after inoculation. The three mice with the largest tumors and the two mice with the smallest tumors were excluded. The remaining 20 mice were randomly divided into four groups (the I\(^{131}\)-mab group, the CD147-mab group, the I\(^{131}\) group and the control group). Animals in the I\(^{131}\) and I\(^{131}\)-mab groups received injections of I\(^{131}\) or I\(^{131}\)-mab at multiple sites (dose: 12.69 μCi/100 μl per mouse); animals in the CD147-mab group received CD147-mab at a dose of 8.82 μM/100 μl per mouse. The animals were injected every other day for a total of five injections. The control mice were injected with saline (100 μl per mouse per injection). After treatment, tumor size was measured weekly, and the following formula was used to calculate tumor volume: “maximum diameter × minimum diameter\(^2\) × 0.5.” The experiment was terminated immediately when the mean tumor volume exceed 2,000 mm\(^3\) in any group, as defined by the Animal Ethics Committee of the Second Military Medical University. At the end of observation, the mice were killed with over-doses anesthesia. The tumor specimens were harvested and weighed, and the tumor tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and immunohistochemically stained.

4.6 | Immunohistochemistry

The sections of paraffin-embedded SMMC-7721 hepatoma xenografts were subjected to streptavidin–peroxidase immunohistochemistry to detect the expression of E-cadherin and p-VEGFR-2. The antibodies used for staining were the...
same as those used for Western blotting. The percentages of positive cells in all sections were determined by counting the cells in five high-magnification fields.

4.7 | Statistical analysis

The cytological experiments were repeated three times independently, and a total of five animals were included in each group. The data are expressed as the mean ± SD. An analysis of variance (ANOVA) and a t test were used for the statistical analysis with the PASW Statistics 18 software. p value less than .05 was considered significant.

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AUTHOR CONTRIBUTIONS

L.W., B.S., X.L. and C.L. conceived the experiments. H.Q., L.C. and C.S. conducted the experiments. L.W., B.S., X.L., Y.Y. and F.S. analyzed the results. All authors reviewed the manuscript.

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