Inhibition of hepatocellular carcinoma cell growth by an anti-insulin-like growth factor-I receptor monoclonal antibody

LU YUE1*, YING WANG2#, HUAMAO WANG3, HUIPING GAO3, JUN LIANG1, AIHUA SUI4, JINYU XIANG1, FANG ZHOU1, CONGCONG XU1, WENWEN ZHAO1, WANHUA LIANG1 and RUYONG YAO9

1Department of Oncology, Affiliated Hospital of Qingdao University, Medical College, Qingdao; 2Shanghai Xuhui Central Hospital, Shanghai; 3State Key Laboratory of Oncogenes and Related Genes, Renji Hospital, Medical School of Shanghai JiaoTong University, Shanghai; 4Central Laboratory, Affiliated Hospital of Qingdao University, Medical College, Qingdao, P.R. China

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Abstract. Hepatocellular carcinoma (HCC) overexpresses insulin-like growth factor-I receptor (IGF-IR), as compared with normal hepatocytes. Since IGF-1R-mediated signaling promotes survival, oncogenic transformation and tumor growth and spread, it represents a potential target for treating HCC. Here, we have generated a murine anti-IGF-1R antibody, 4F2, that recognizes the IGF-IRα subunit and blocks in vitro IGF-I and IGF-II-induced cell proliferation of SMMC-7721 and Bel-7402 HCC cell lines. 4F2 can inhibit IGF-1R auto-phosphorylation, IRS-1 phosphorylation and the activation of the major downstream signaling molecules AKT and mitogen-activated protein kinase. Additionally, we observed a moderate increase in apoptosis as demonstrated by detection of changes in the expression of the pro-apoptotic and anti-apoptotic proteins Bax and Bcl-2 after 4F2 treatment. Combined treatment with 4F2 and doxorubicin was more effective in reducing cell proliferation and promoting apoptosis than either agent alone. These data support that therapeutic anti-IGF-IR antibodies are potential new agents for treating HCC.

Introduction

The insulin-like growth factor receptor-I (IGF-IR) is a membrane-bound tyrosine kinase receptor that plays an important role in tumor cell proliferation, differentiation, apoptosis and metastasis (1,2). IGF-IR can bind with both IGF-I and IGF-II and is overexpressed in some cancers (3-8). The expression of IGF-IR was constitutively low in normal hepatocytes, but highly expressed in HCC and HCC cell lines (9). Epidemiologic data have shown that elevated plasma IGF-I level is linked with prostate, breast, lung and colon cancer risk (10,11). IGF-II is frequently overexpressed in liver cancer (12-17). The level of IGF-II is highly expressed in many human malignancies, such as breast cancer, pediatric tumors, colon cancer and hepatocellular carcinoma (HCC) (18,19). Binding of IGF-II with IGF-IR has been associated with increased tumor cell mitosis and anti-apoptosis as well as enhanced angiogenesis (20). Therefore, IGF-IR is an attractive antitumor target for HCC.

Mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3/kinase/AKT are the principle pathways for transduction of the IGF signal (21,22). After ligand-dependent receptor autophosphorylation, IGF-IR phosphorylates a series of adaptor proteins, such as insulin receptor substrate-1 (IRS-1), to activate intracellular signaling pathways. The MAPK pathway plays an important role in the mitogenic signal elicited after IGF stimulation but may also function in cell survival in cells overexpressing the IGF-IR (23,24). After IGF stimulation the phosphatidylinositol 3/kinase elicits survival processes including the activation of the Akt and, as a result, has been shown to protect cells from damage-induced apoptosis (25).

The development of specific small molecule inhibitors of IGF-IR tyrosine kinase activity was challenging because of the high degree of homology to insulin receptor. Recently, many neutralizing antibodies specific for IGF-IR have been developed such as α-IR3 (26), scFv-FC (27), CP-751,871 (28) and IMC-A12 (29). Although the antitumor effects of these antibodies were tested for several cell types in preclinical studies, no comprehensive research regarding the antitumorigenic impact on HCC cells have been reported to date. Here, we generated a murine anti-IGF-IR antibody to test our hypothesis both in vitro and in vivo by treating HCC tumor cells with the 4F2 antibody alone and in combination with the cytotoxic chemotherapeutic drugs. The mechanism underlying the antitumor effect of 4F2 was also elucidated.
Materials and methods

Cell lines and culture. The human HCC cell lines SMMC-7721, 7402 and breast cancer cell lines MCF-7, MDA-MB-468 were cultured in DMEM medium containing 10% fetal bovine serum, NIH3T3 cells overexpressing IGF-IR, NIH3T3/IGF-IR were constructed by our laboratory and grown in DMEM supplemented with 10% fetal bovine serum.

Reagents. Human recombinant IGF-I and IGF-II were purchased from Calbiochem. Annexin V-FITC apoptosis detection kit I was from BD Biosciences. Specific antibodies against the following antigens were used: IGF-IRβ (C20), insulin receptor (C19), IGF-IRα (3B7), phospho-Y1158/1162/1163 IGF-IR, ERK1,2, AKT, phospho-s473 AKT, phospho-p4/2 p44 extracellular signal-regulated kinase (ERK), bel-2, p27, CyclicD1 (Santa Cruz Biotechnology); IRS-1, phospho-Y896, IRS-1, Bax, Bad (Epitomics); 4G10 (Up-state Biotechnology). Anti-mouse and anti-rabbit horseradish peroxidase conjugates were from Amersham. Cell Counting Kit-8 was from Dojindo Laboratories.

Generation of anti-IGF-IR antibodies. BALB/c mice were immunized i.p. with human IGF-IR overexpressing 3T3-IGF-IR cells (5x10^6 cells, suspended in 0.2 ml of PBS) with Freund's adjuvant. Some of the animals were boosted with 10^6-10^7 cells several times before fusion. The splenocytes from immunized mice were isolated and used to generate hybridoma according to standard protocols (30). The hybridoma supernatants were screened by ELISA for specific binding to the 3T3-IGF-IR cells used for immunization and for the absence of binding to 3T3 cells. Immulon-2HB plates (Dynatech) precoated with 100 µl of phytohemagglutinin lectin (20 µg/ml; Sigma) were charged with trypsin/EDTA treated cells (1-3x10^6 cells/100 µl), centrifuged, then kept at ambient temperature for 10 min and finally dried overnight at 37°C. The wells were blocked with 5 mg/ml BSA in PBS (blocking solution) for 1 h at 37°C, washed gently with PBS, and then incubated with supernatants from hybridoma clones (diluted in blocking solution) for 1 h. The wells were washed with PBS, incubated with goat anti-mouse-IgG-Fc-antibody-HRP conjugate (0.8 µg/ml; in blocking solution) for 1 h, washed, and the binding was detected using ABTS/H_2O_2 substrate [0.5 mg/ml ABTS, 0.03% H_2O_2, 0.1 M citrate buffer (pH 4.2); 405 nm]. A hybridoma supernatant was identified, which showed strong binding to 3T3-IGF-IR cells and not to 3T3 cells. Using protein A affinity chromatography, a murine IgG2a antibody was isolated from the hybridoma supernatant and designated 4F2.

RT-PCR. Total RNA was extracted from cultured cell lines with RNA Clean following the recommendation of the manufacturer (Hybaid, London, UK). To eliminate any possible contamination with genomic DNA, RNAs were treated with 1 unit DNase per mg RNA for 15 min at room temperature and were then reverse transcribed into cDNA using oligo(dt) primers and the SuperScript Preamplification-Kit following the manufacturer's instructions (Gibco). PCR reactions were carried out in a total volume of 50 µl containing 400 nM of each primer, 200 mM of each dNTP (Pharmacia, Uppsala, Sweden), 50 mM KCl, 1.5 mM MgCl_2, 10 mM Tris and 1 unit Taq-polymerase (Pharmacia). The primers for IGF-IR were 5'-GGGAAATGGAGTGTGTTATG-3' (forward) and 5'-CACA GAAGCTTCTGATGAA-3' (reverse). Amplification of human β-actin served as an internal control. The primers used were 5'-GGACCTGACTGACTACCTC-3' (forward) and 5'-TCATA CTCTGCTTCGCT-3' (reverse).

Immunocytochemistry labeling and microscopy. The cellular localization of proteins of interest was accomplished by indirect immunocytochemistry. Briefly, SMMC-7721, Bel-7402 and 3T3-IGF-IR cells were plated on sterile glass cover slips in 6-well plates and allowed to attach overnight. Cells were then rinsed twice in PBS, fixed in 4% phosphate-buffered paraformaldehyde for 15 min, and permeabilized in acetone at -20°C for 4 min. Following permeabilization, cells were blocked in 5% normal goat serum-PBS for 30 min, incubated with a primary antibody against IGF-IR for 1 h at room temperature, washed thrice in PBS, and then incubated with goat anti-mouse secondary antibodies. Cells were then incubated 1 h with peroxidase-anti-peroxidase mix and rinse with buffer as before. Then cells were incubated in fresh DAB solution (10 mg DAB + 20 µl 38% H_2O_2 in 20 ml 0.1 M Tris pH 7.2; 200 µl 1 mM imidazole), The reaction was stopped by washing in water when a uniform brown color first became visible on the cells. Microscopic analyses were done using a Leica 4000B microscope in accordance with established methods.

Immunoprecipitation and western blot analysis. Cells were plated into 6-well culture dishes and grown to 70-80% confluency. Monolayers were washed twice in PBS and cultured overnight in serum-free medium. Antibody was then added in fresh serum-free medium and incubated at 37°C for 2 h. Cells were incubated with ligand for 15 min and then placed on ice and washed with ice-cold PBS. For immunoprecipitation of pure IRs, IGF-IR-depleted supernatant from an IGF-IR immunoprecipitate was immunoprecipitated with anti-insulin antibody C19 or 4F2. To isolate pure IGF-IRs, IR-depleted supernatant from an IR immunoprecipitate was immunoprecipitated with anti-IGF-IR antibody C-20 or 4F2. Immunoprecipitates bound to the protein A-agarose beads were stripped into denaturing gel sample buffer. Lysates or immunoprecipitates were processed for denaturing gel electrophoresis and run on a 10% SDS-PAGE, and blotted to nitrocellulose membrane by western blotting.

Cell proliferation/survival assays. The effect of 4F2 treatment on the growth of human HCC cell lines SMMC-7721, Bel-7402 upon stimulation by IGF-I, IGF-II or serum was measured using the CCK-8 assay after 3 days. Typically, 1500-3000 cells/well were plated in a 96-well plate in regular growth medium with serum, which was replaced with serum-free medium the next day. After 1 day of incubation in serum-free medium, the cells were washed gently with serum-free medium and then incubated with 4F2 antibody and doxorubicin alone or combination in serum-free medium for 2 h, which was followed by the addition of IGF-II solution (or IGF-I solution or serum) to obtain a final concentration of 5-50 µg/ml 4F2 antibody and 20 ng/ml IGF-II (or 20 ng/ml IGF-I or 2% serum). The cells were then allowed to grow for 3 days. Of the CCK-8 solution 10 µl was added to cells cultured for the designated time. The plates were incubated for 1-4 h in the incubator. The resulting

4F2
color was assayed at 450 nm using a microplate absorbance reader (Tecan, Safire II, Switzerland).

Inhibition of IGF-IR-mediated cell signaling by 4F2. The potential of 4F2 to inhibit the IGF-I-stimulated autophosphorylation of IGF-IR and the phosphorylation of downstream effectors, IRS-1, Akt and ERK, was studied in SMMC-7721 cells. Antibodies were added to cells for 2 h. Cells were then stimulated with 20 ng/ml IGF-I or IGF-II for 15 min at 37˚C, washed twice with cold PBS containing 0.1 mmol/l sodium vanadate and lysed in lysis buffer [50 mmol/l HEPES (pH 7.4), 150 mmol/l NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/l MgCl₂, protease inhibitors, 2 mmol/l sodium vanadate]. Lysates were incubated on ice for 30 min and then centrifuged at 13000 rpm for 10 min at 4˚C. Protein concentrations of the lysates were measured with BCA kit (Pierce). Lysates were then subjected to immunoprecipitation and western blot analysis.

Receptor degradation analysis. SMMC-7721 cells were plated in regular culture medium followed by overnight incubation in serum-free medium. IGF-I, IGF-II (20 ng/ml) or 4F2 was then added and cells were incubated at 37˚C for up to 24 h. Cells were washed in ice-cold PBS, lysed in immunoprecipitation assay buffer, and quantitated by BCA kit (Pierce). Equal amount of cell lysates was separated on 10% SDS-PAGE, transferred to nitrocellulose filters, probed with an anti-IGF-IR rabbit polyclonal IgG and revealed with an anti-rabbit IgG coupled to the HRP and visualized by ECL.

Apoptosis assays. SMMC-7721 cells were treated with 4F2 or doxorubicin in the presence of IGF-II (or serum) for 24 h. Apoptotic and necrotic index were assessed by flow cytometry, using fluorescein isothiocyanate (FITC) labeled Annexin V, and simultaneously with PI stain. Cells were washed twice with ice-cold PBS and incubated for 30 min in a binding buffer (1 μg/ml PI and 1 μg/ml FITC labeled Annexin V), respectively. FACS analysis for Annexin V and PI staining was performed by flow cytometry. All experiments were performed in triplicate.

Statistical analysis. Student’s t-test and One-way ANOVA analysis were performed for continuous variables. The χ² test or Fisher’s exact test were used for categorical variables. The error bars represent the standard error of the mean. Statistical significance for all the tests, assessed by calculating P-value, was <0.05 from two-sided tests. The statistical analyses were performed using SAS 9.0 software (SAS Inc., Cary, NC, USA).

Results

Identification of an inhibitory anti-IGF-IR monoclonal antibody through a rapid biological screen. To generate monoclonal antibodies, BALB/c mice were immunized and subsequently boosted with 3T3-IGF-IR cells that overexpress human IGF-IR. The generated hybridoma supernatants were screened by cell-based ELISA methods for specificity of binding and biological activity. The hybridoma clones that bound to the 3T3-IGF-IR cells but not to the NIH-3T3 cells were selected. The selected antibody clones were further screened by their growth, inhibiting activity on HCC cells using CCK-8 assay (data not shown). As the most effective inhibitory antibody clone, 4F2 was picked out for further study.

Expression of IGF-IR in hepatocellular carcinoma. mRNA expression of the insulin-like growth factor receptor 1 (IGF-IR) was investigated in the human hepatoblastoma and hepatocellular carcinoma cell lines. Robust expression of mRNAs specific for IGF-IR was detected in all cell lines (Fig. 1A). To evaluate the protein expression of IGF-IR, western blot analysis was performed. Both the IGF-IR precursor and
the β-chain of the mature form of IGF-1R were detected in HCC cells (Fig. 1B). Immunocytochemistry results show that most IGF-IR located in the cell membrane and cytoplasm of SMMC-7721 and Bel-7402 cells.

**Specificity identification of 4F2.** Since IGF-IR shares considerable homology with the IR (34), it is necessary to demonstrate the selectivity of 4F2 for the IGF-IR. We performed successive immunoprecipitation of IGF-IR and IR in MDA-MB-468 and MCF-7 cell lines, which possess a greater IGF-IR:IR and IR:IGF-IR ratio, respectively (31), to obtain purified classical IGF-IR and IR homodimers. Immunoprecipitation and immunoblot analysis showed that 4F2 bound selectively to IGF-IR but not to IR (Fig. 2A and B). To demonstrate selective blocking effect on IGF-IR signaling but not that of IR, we performed successive immunoprecipitation after cells were treated with IGF-I and insulin stimulation. Immunoblot analysis showed that 4F2 completely inhibited the phosphorylation of IGF-IR but not that of IR (Fig. 2C and D).

**4F2 inhibits the proliferation of HCC cells.** To characterize the in vitro antiproliferative effect of 4F2, HCC cell lines were grown in serum-free medium with exogenously added IGF-I/IGF-II in the presence or absence of 4F2, typically for 3 days, and the cell viability was then measured by CCK-8 assay. We found a dose-dependent reduction in the number of viable cells (Fig. 3). In similar experiments based on the CCK-8 assay after 3 days of growth, 4F2 treatment caused dose-dependent inhibition of the serum (2%)-stimulated proliferation and survival of SMMC-7721 and Bel-7402 cells, implicating the essential role of IGF-I as a growth factor present in serum (Fig. 3).

**Antineoplastic potency of 4F2 in combination with the doxorubicin.** Several studies have reported that combinations of doxorubicin and small molecular inhibitor of IGF-IR resulted in additive growth inhibitory effects on HCC cells (36). We studied possible (over-) additive antineoplastic effects of 4F2 plus doxorubicin in HCC cells. Cells were treated with 4F2 (20 µg/ml) alone or in combination with rising concentrations of the doxorubicin (50-250 nM) for 72 h. Upon treatment with doxorubicin alone, a dose-dependent growth inhibition was observed after 72 h in SMMC-7721 (Fig. 4A) and Bel-7402 cells (Fig. 4B), 250 nM of doxorubicin caused a 51 and 34% inhibition in SMMC-7721 and Bel-7402 cells, respectively (P<0.01). The antineoplastic effects of doxorubicin increased when the doxorubicin was combined, approximately 69 and 58% inhibition was observed when 20 µg/ml 4F2 was combined with 250 nM doxorubicin in SMMC-7721 and Bel-7402 cells (P<0.01). Combinatorial treatment with 4F2 and doxorubicin resulted in significantly higher inhibitory effects on SMMC-7721 and Bel-7402 cells than treatment with 4F2 (P<0.001) or doxorubicin (P<0.05) alone.

The anti-IGF-IR therapeutic antibody 4F2 blocks IGF-induced IGF-IR signaling and downregulates the expression of IGF-IR in HCC cells. The effect of 4F2 on the IGF-I-stimulated or
IGF-II-stimulated activation of IGF-IR and the downstream signaling were then measured. Serum-starved SMMC-7721 cells were treated for 2 h with various concentrations of 4F2 and then stimulated with IGF-I or IGF-II in serum-free medium for 15 min. The cells were then lysed and the lysates were analyzed for the phosphorylation levels of effector proteins in the IGF-IR-signaling pathway, including IGF-IR, IRS-1, Akt and MAPK. At a concentration of 50 µg/ml, 4F2 completely inhibited both IGF-I-induced and IGF-II-induced phosphorylation of IGF-IR (Fig. 5A). In addition, at concentrations of 20 and 50 µg/ml, 4F2 inhibited IGF-I-induced and IGF-II-induced phosphorylation of IRS-1, Akt and MAPK (Fig. 5A). To evaluate the potential downregulation of IGF-IR by 4F2 treatment, the level of IGF-IRβ chain in 4F2-treated SMMC-7721 cells was assessed by western blotting. No detectable decrease in the amount of IGF-IRβ chain was seen during the first 4 h of treatment with 4F2 in Bel-7402 and SMMC-7721 cells, but a modest downregulation of the receptor was observed after 48-h treatment (Fig. 5B). In contrast, no downregulation of IGF-IRβ was observed upon treatment with the ligand IGF-I and IGF-II.

4F2 enhances the induction of apoptosis by doxorubicin. The CCK-8 assays above show that 4F2 combined with doxorubicin additively inhibits HCC cells growth but does not characterize the mechanism of cell death. To examine whether 4F2 enhanced cytotoxicity of doxorubicin to cancer cells was mediated through increased apoptosis, Annexin V/PI staining was used to examine the apoptosis in SMMC-7721 cells. From the statistical analysis of cytometry data, we found that doxorubicin combined with 20 µg/ml 4F2 induced more apoptotic cells (30.4±1.56%, P<0.01) than doxorubicin alone (17.1±1.25%, Fig. 6). In order to determine whether the increased cytotoxicity reflected induction of apoptosis, Annexin V binding and propidium iodide staining were carried out using the Annexin V-FITC Apoptosis Detection Kit 1 (BD Pharmingen) and flow cytometry. As show in Fig. 6, combination of 20 µg/ml 4F2 and doxorubicin resulted in a nearly 2-fold increase in apoptosis relative to control. The combination seemed to result in a more effective induction of apoptosis than doxorubicin alone.

Figure 3. Potent inhibition of proliferation and survival of cancer cells by 4F2. Cancer cells were treated with 4F2 and control antibody in the presence of IGF-I, IGF-II or serum, and the proliferation of cells was monitored using CCK-8 assay (after 3 days). (A) and (B) inhibition of IGF-I, IGF-II or serum-stimulated proliferation of HCC cells SMMC-7721 and Bel-7402 by different concentration of 4F2 and control antibody.

Figure 4. Antiproliferative effects of 4F2-based combination treatments. HCC cells (A) SMMC-7721 and (B) Bel-7402 were treated for 72 h with rising concentrations of the doxorubicin alone or in combination with 20 µg/ml 4F2 and effects of 20 µg/ml 4F2 alone. Means ± SEM, of three to five independent experiments.
in additivity of the rates of apoptosis induced by each single drug at the concentrations and time point tested.

4F2 regulates expression of apoptosis-specific and cell cycle regulating proteins. To elucidate the signaling pathways modulated by 4F2 inhibition in HCC cells, we investigated changes in the expression of apoptosis-specific proteins and cell cycle regulating proteins. Cells were incubated with IGF-I/II in the presence or absence of 4F2 for 48 h. 4F2 dose-dependently downregulated anti-apoptotic Bcl-2 expression while upregulated pro-apoptotic Bax expression. Moreover, 4F2 downregulated cyclin D1 expression while upregulated p27 expression (Fig. 7).

Discussion

Several reports indicate that IGF-IRs are expressed frequently in HCC (32), most likely leading to the aggressive growth characteristics of tumors. Consequently, the IGF-IR is a prom-
ising target for innovative treatment strategies in HCC. In the present study, we have generated a murine anti-IGF-1R antibody 4F2 which specifically recognizes IGF-IR but not insulin receptor. This selectivity may be an advantage over potential IGF-IR kinase inhibitors which may partly inhibit insulin receptor and induce hyperglycemia. By blocking IGF-IR activation, 4F2 can effectively inhibit its signal transduction. The relative contribution of these effects to the anticancer activity of 4F2 in different settings (including in vivo models) remains to be determined. To elucidate the underlying mechanisms of the 4F2 antiproliferative activity on HCC cells, the expression of apoptosis and cell cycle related proteins were also examined. Compared with untreated cells, 4F2 dose-dependently downregulated anti-apoptotic Bcl-2, pro-apoptotic Bax was upregulated. Moreover, cell cycle promoting cyclin D1 was downregulated, while the cell cycle arresting p27 was upregulated by 4F2.

Since IGF-IR signaling has been shown to prevent tumor cells from the cytotoxic effects of chemotherapy and may play an important role in tumor cell drug resistance (33-35), we supposed that anti-IGF-IR antibody 4F2 may be combined with chemical drug for treating HCC. We then studied whether inhibition of IGF signaling alters chemosensitiveness, the results showed that combined treatments using 4F2 with conventional cytotoxic agent doxorubicin, significantly increase the cytotoxic effects, suggesting that this combination might offer an alternative strategy for treating HCC. As we all known, chemotherapeutic drugs inhibit cancer cell growth mainly by inducing cell apoptosis. We also know that activated IGF-1R is a powerful inhibitor of apoptosis (36,37). For instance, Dunn et al (38) reported that IGF-I could induce a 20-40% increase in cell survival of breast cancer cells treated with anticancer drugs. A study have proved that when the novel IGF-1R tyrosine kinase inhibitor NVP-AEW541 was combined with cytotoxic chemotherapy, additive antiproliferative effects were observed in HCC, Furthermore, combinatorial treatment with IGF-1R tyrosine kinase inhibitor NVP-AEW541 and cytotoxic drugs impose additive antiproliferative effects on HCC. However, the development of specific small molecule inhibitors of IGF-IR tyrosine kinase activity was challenging because of high degree of homology to insulin receptor. Because of the high specificity of 4F2 in recognizing IGF-1R, we think it may be a new substitute of TKI in combination with chemotherapeutic drug for treating HCC. We observed that 4F2 actually sensitizes SMMC-7721 cell lines to doxorubicin, mainly by inducing apoptosis as shown in Fig. 6.

IGF-IR is a promising anticancer therapy target because of its defined role in establishing and maintaining the cancer phenotype. Our study provides first evidence that the growth of human hepatocellular SMMC-7721 cells can be potently suppressed by IGF-IR inhibition with anti-IGF-IR antibodies. Evidence suggesting a link between IGF-IR signaling and resistance to cytotoxic therapies provides rationale for combining IGF-IR inhibitors with chemotherapy. The present study is the first published report showing a favorable interaction between chemotherapy and IGF-IR blockade with an anti-IGF-IR monoclonal antibody. Anti-IGF-IR antibodies are therefore promising agents as monotherapy or in combination therapy for HCC.

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