Cancer Letters 342 (2014) 113–120

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

The monoclonal antibody CH12 augments 5-fluorouracil-induced growth suppression of hepatocellular carcinoma xenografts expressing epidermal growth factor receptor variant III

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ARTICLE INFO

Article history:
Received 19 April 2013
Received in revised form 16 August 2013
Accepted 26 August 2013

Keywords:
Epidermal growth factor receptor variant III
Hepatocellular carcinoma
mAb CH12
5-Fluorouracil
Combination therapy

ABSTRACT

5-Fluorouracil (5-FU) is one of the most common chemotherapeutic agents used for the treatment of hepatocellular carcinoma (HCC). However, chemoresistance has precluded the use of 5-FU alone in clinical regimens. Combination therapies with 5-FU and other anticancer agents are considered to be a therapeutic option for patients with HCC. We previously reported that the expression of epidermal growth factor receptor variant III (EGFRvIII) can decrease the sensitivity of HCC cells to 5-FU. To overcome this problem, in this study, we elucidated the mechanism underlying EGFRvIII-mediated 5-FU resistance. We observed that EGFRvIII expression can induce miR-520d-3p downregulation and the ensuing upregulation of the transcription factor E2F-1 and the enzyme thymidylate synthase (TS), which may lead to drug resistance. Intriguingly, we found that CH12, a monoclonal antibody directed against EGFRvIII, and 5-FU together had an additive antitumor effect on EGFRvIII-positive HCC xenografts and significantly improved survival in all mice with established tumors when compared with either 5-FU or CH12 alone. Mechanistically, compared with 5-FU alone, the combination more noticeably downregulated EGFR phosphorylation and Akt phosphorylation as well as the expression of the apoptotic protector Bcl-x_L and the cell cycle regulator cyclin D1. Additionally, the combination upregulated the expression of the cell cycle inhibitor p27 in *in vivo* treatment. More interestingly, CH12 treatment upregulated miR-520-3p and downregulated E2F-1 and TS at the mRNA and protein levels. Collectively, these observations suggest that the combination of 5-FU with mAb CH12 is a potential means of circumventing EGFRvIII-mediated 5-FU resistance in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common forms of cancer in the world and is the third leading cause of cancer-related death [1]. Current treatment strategies, such as liver transplantation, surgical resection, or regional therapy for advanced HCC, are unsatisfactory. Chemotherapy is commonly used for the treatment of various malignancies. However, systemic cytotoxic chemotherapeutic agents have not significantly improved the survival of HCC patients because of the resistance of HCC to anticancer drugs [2,3]. Tumor recurrence after curative liver resection remains high, and most patients die within several months of diagnosis [4].

Because of its significant cell-killing activity, 5-fluorouracil (5-FU) has been the most commonly used chemotherapeutic agent against HCC. However, similar to other anticancer agents, chemoresistance is a major clinical obstacle to successful 5-FU chemotherapy for HCC patients. Chemoresistance has precluded single use of 5-FU in clinical settings [5]. Thus, a variety of combination chemotherapy regimens have been studied in HCC. Some previous studies reported that use of a combination of 5-FU and other anticancer agents, such as cisplatin, interferon, doxorubicin and vitamin K2, showed superior response rates among patients with HCC [5–8]. However, most of these regimens have not been validated in large randomized phase III studies. Moreover, combination chemotherapy regimens have greater toxicity than single agents [3]. Therefore, it is urgently needed to find a new effective therapeutic strategy for patients with HCC.

Abbreviations: EGFR, epidermal growth factor receptor; EGFRvIII, epidermal growth factor receptor variant III; HCC, hepatocellular carcinoma; 5-FU, 5-fluorouracil; DPYD, dihydrouracil dehydrogenase; OPRT, orotate phospho ribosyltransferase; TS, thymidylate synthase.

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http://dx.doi.org/10.1016/j.canlet.2013.08.038

Recently, the epidermal growth factor receptor (EGFR) was successfully targeted for cancer therapy [9]. EGFR expression in HCC has been reported [10]. Several drugs that target EGFR have been developed, including an EGFR monoclonal antibody (cetuximab) [11,12]. Unfortunately, no obvious responses were observed in HCC patients after treatment with cetuximab [13]. The presence of EGFR gene mutations may result in a limited clinical response to EGFR-targeting therapies in HCC patients. The most common EGFR variant is EGFRvIII (also referred to as de2-7 EGFR), which can promote tumor cell growth both *in vitro* and *in vivo* [14,15]. This variant is not present in normal tissue but has been detected in multiple human malignancies, such as glioma, non-small cell lung carcinoma, breast cancer, head and neck cancer, ovarian carcinoma and HCC [15–19]. Recently, we also observed EGFRvIII expression in liver cancer cell lines, and we found that its expression can decrease the sensitivity of HCC cell lines to 5-FU [20]. CH12, an anti-EGFRvIII monoclonal antibody developed in our laboratory, can preferentially bind to EGFRvIII and significantly inhibit the growth of Huh7-EGFRvIII and SMMC-7721 xenografts *in vivo* with a growth inhibition ratio much higher than that of cetuximab [21].

Therefore, we hypothesized that CH12 may enhance the 5-FU-mediated growth inhibition of HCC xenografts expressing EGFRvIII, and we found that combining 5-FU with CH12 caused a marked and additive anti-proliferative effect both *in vivo* and *in vitro*. Furthermore, we assessed the effects of CH12 on the expression of enzymes that function in 5-FU metabolism, including dihydrouracil dehydrogenase (DPYD), orotate phosphoribosyltransferase (OPRT), and thymidylate synthase (TS), to gain insights into the mechanism underlying the additive effect of combination therapy with 5-FU and CH12.

2. Materials and methods

2.1. Cell culture

The human hepatocellular carcinoma cell lines Huh7-EGFRvIII (Huh-7 cells expressing exogenous EGFRvIII [20]), Huh7-DK (Huh-7 cells expressing DK (a kinase-deficient mutant of EGFRvIII [22])) and SMMC-7721 (Chinese Academy of Science, Shanghai, China) were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.2. Western blot analysis

Cell lysates were collected and centrifuged for 10 min at 12,000 rpm. The supernatant was transferred to a clean tube, and proteins were quantified using the BCA Kit (Pierce, Rockford, IL). Proteins (40 μg) were separated on 10% SDS–PAGE gels and transferred to nitrocellulose membranes (Millipore Billerica, MA).The membranes were blocked with 5% skim milk and incubated overnight at 4 °C with primary antibodies. The following antibodies were used: mAb 12H23, anti-phospho-EGFR (Tyr1068) (Abcam, Cambridge, United Kingdom) and anti-GAPDH (Kang-Chen Bio-tech, Shanghai, China) antibodies. The anti-DPYD, anti-OPRT, anti-TS, anti-E2F-1, anti-phospho-Akt (Thr308), anti-Akt, anti-Bcl-x_L, anti-cyclin D1, and anti-p27 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The immune complexes were detected through incubation of the membrane with horseradish peroxidase-conjugated goat anti-mouse antibody, goat anti-mouse (Shanghai Raygene Biotechnology, Shanghai, China) or donkey anti-goat antibody (Kang-Chen Bio-tech, Shanghai, China) for 1 h at room temperature and subsequent exposure of the membrane to enhanced chemiluminescence reagents (Pierce, Thermo Scientific, Rockford, IL).

2.3. Reverse transcription and quantitative real-time PCR analysis

Total RNA was extracted from cell lines with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Complementary DNA (cDNA) of genes was synthesized using the ImProm-II™ Reverse Transcription Kit (Promega, Madison, WI) using 1 μg of total RNA as a template. cDNA of miRNA was synthesized using the Prime-Script RT Reagent Kit (TaKaRa, Dalian, China) and a reverse transcription primer from RiboBio (RiboBio, Guangzhou, China). The expression of DPYD, OPRT, TS, E2F-1 and miR-520d-3p was assessed *via* quantitative real-time PCR analysis using a Takara SYBR Premix Ex Taq™ Reagent Kit (TaKaRa, Dalian, China) and an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' protocols. The primers used are as follows: DPYD forward primer: 5′-CGTCCTCCAGGTATGCAGTG-3′; DPYD reverse primer: 5′-GGCAGTTTCTTGTCCATGAGTT-3′; OPRT forward primer: 5′-CTAGAGCACCGGTTAGAATGG-3′; OPRT reverse primer: 5′-GATTATCTCCTCCTGCTTCCAA-3′; TS forward primer: 5′-CCAAGAGATC TTCCTCTGATGG-3′; TS reverse primer: 5′-GTTCTCGCTGAAGCTGAATTTT-3′; E2F-1 forward primer: 5′-GGACTCTTCGGAGAACTTTC AG-3′; E2F-1 reverse primer: 5′-ACAACAGCGGTTCTTGCTC-3′;
β-actin forward primer: 5′-CCTGGCACCCAGCACAAT-3′; β-actin reverse primer: 5′-GGGCCGGACTCGTcATACTG-3′. Amplification was performed using the following conditions: one cycle of 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s using 1 × SYBR® Premix Ex Taq™ II, 0.4 μM of each primer, 1 × ROX Reference Dye II, and 2 μl of cDNA template. β-actin served as an internal control. For miRNA detection, U6 small nuclear RNA was used for normalization. The transcript levels were determined using the comparative threshold cycle method [23]. All samples were run in triplicate.

2.4. MicroRNA array analysis

Small RNA with a miRNA-rich fraction was prepared using miRNA Isolation Kit (Ambion, Austin, TX). miRNA microarray was performed as described in details on the website of CapitalBio (http://www.capitalbio.com). Briefly, Fluorescein-labeled miRNAs were used for hybridization on each miRNA microarray chip containing human miRNA probes in triplicate. Raw data were normalized and analyzed in GenePix Pro 4.0 software (Axon Instruments, PA). Statistical comparisons were done with SAM analysis. The microarray data were confirmed through TaqMan microRNA assays (Applied Biosystems, Foster City, CA).

2.5. Transfection of miR-520d-3p mimics or antagomiR

Cells were transfected with miR-520d-3p mimics or antagomiR and the mock (non-related mimic or antagomir) (Ribobio, Guangzhou, China) using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). For western blot assays, cells were collected 72 h after transfection.

2.6. In vitro cell proliferation assay

The effect of the test agents on cell viability was assessed with the CCK-8 Assay Kit (Dojindo Laboratories, Rockville, MD). The cells were plated in triplicate in 96-well plates at densities of 2×10^3 cells per well. After 24 h, the cells were placed in complete medium containing the indicated concentrations of drugs or vehicle control. After 72 h, cell proliferation was measured using the CCK-8 Kit. CCK-8 solution (10 μl) was added to 100 μl of culture media, and the optical density was measured at 450 nm. Three independent experiments were performed.

2.7. In vivo antitumor effects

Huh7-EGFRvIII cells (3×10^6) were subcutaneously injected into 4–6-week-old nude mice. When tumors had reached a mean tumor volume of 150 mm^3, mice were randomly allocated into six groups ($n = 6$) and assigned to receive one of the following four injections: (i) vehicle (sterile PBS); (ii) 25 mg/kg 5-fluorouracil dissolved in sterile saline; (iii) 12.5 mg/kg mAb CH12 in sterile saline; (iv) 25 mg/kg 5-fluorouracil and 12.5 mg/kg mAb CH12 in sterile saline. Injections were administered intraperitoneally three times per week for 2 weeks. Tumor volumes were measured every other day in two dimensions with vernier calipers. Tumor volumes were calculated using the formula, [length × (width^2)] × 0.5. Thirty-nine days after tumor cells inoculation, the mice were anesthetized and sacrificed by cervical dislocation. The tumors were surgically excised and weighed. Data were expressed as percent inhibition of tumor growth. Tumor tissues from the *in vivo* experiments were collected for Western blot analysis and immunohistochemical studies. Mice were manipulated and housed according to protocols approved by the Shanghai Medical Experimental Animal Care Commission.

2.8. Immunohistochemical staining

All studies were conducted as previously described [21]. Briefly, anti-Ki-67 (Santa Cruz Biotechnology) was used to assess cell proliferation on formalin-fixed paraffin-embedded tumor tissues. The slides were visualized using a diaminobenzidine staining kit (Tiangen Biotech, Beijing, China) and counterstained in hematoxylin. The percentage of Ki-67 positive tumor nuclei in 6 randomly selected ×400 fields of three representative mice from each group was determined by examining the ratio of labeled nuclei: total nuclei to give proliferation indexes.

2.9. TUNEL assay

To quantify apoptosis, the deparaffinized and rehydrated tumor tissues sections were assayed using a TUNEL assay kit (Beyotime Biotechnology, Nanjing, China). All the sections were observed and photographed with an Axioskop 2 microscope (Carl