Autophagy Activation in Hepatocellular Carcinoma Contributes to the Tolerance of Oxaliplatin via Reactive Oxygen Species Modulation

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Abstract

Purpose: Understanding the roles of mammalian autophagy in cancer highlights recent advances in the pharmacologic manipulation of autophagic pathways as a therapeutic strategy for cancer. However, autophagy status and corresponding functions in hepatocellular carcinoma (HCC) after therapeutic stress remain to be clarified. This study was to determine whether the autophagic machinery could be activated after chemotherapy and the contribution of autophagy to tolerance of oxaliplatin in HCC.

Experimental Design: Autophagy activation and cell death induced by oxaliplatin were examined in two HCC cell lines as well as in vivo using an HCC model in nude mice. HCC tissue samples with or without locoregional chemotherapy before surgery were also examined by immunohistochemical and electron microscopic analysis.

Results: Autophagy was functionally activated in HCC cell lines and xenografts after oxaliplatin treatment. Suppression of autophagy using either pharmacologic inhibitors or RNA interference of essential autophagy gene enhanced cell death induced by oxaliplatin in HCC cells. Generation of reactive oxygen species has an important role in the induction of cell death by oxaliplatin in combination with autophagy inhibitors. Critically, the combination of oxaliplatin with autophagy inhibitor chloroquine resulted in a more pronounced tumor suppression in HCC xenografts. Furthermore, autophagy-specific protein LC3 and autophagic autophagosome formation were induced to a significantly higher level in HCC specimens that had been subjected to locoregional chemotherapy.

Conclusions: Autophagy activation under therapy stress contributes to HCC tumor cell survival. Targeting the autophagy pathway is a promising therapeutic strategy to enhance the effects of chemotherapy and improve clinical outcomes in HCC patients. Clin Cancer Res; 17(19); 6229–38. ©2011 AACR.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the leading causes of cancer-related mortality (1). Surgery is the form of treatment that offers the greatest potential for cure, but most patients have unresectable disease at presentation. Conventional systemic chemotherapy options have typically yielded poor outcomes for these patients (2). With the development of regional cancer therapy and multimodality treatments, localized unresectable large HCCs have been converted to resectable small HCCs (3). Locoregional chemotherapy, such as hepatic arterial infusion chemotherapy, is commonly used as a nonsurgical approach for downstaging; however, it does not have the desired effect in most treated HCCs. Moreover, residual tumor cells after chemotherapy may have a more aggressive behavior that promotes metastasis (4). Even in patients who undergo radical resection, a majority develop recurrent disease by 5 years (5). Therefore, there is an urgent need for improved antitumor drugs for advanced or recurrent HCC.

Understanding the roles of mammalian autophagy in cancer has led to recent advances in the pharmacologic manipulation of autophagic pathways as a therapeutic strategy for cancer (6). Autophagy is an evolutionarily conserved process that involves lysosomal degradation of cytoplasmic and cellular organelles. Although cell death resulting from progressive cellular consumption has been attributed to unrestrained autophagy, which led to the
Oxaliplatin was dissolved in 100% dimethyl sulfoxide (DMSO). Compounds and corresponding functions in HCCs after use of these therapies including DNA-damaging agents, targeted therapies (e.g., tyrosine kinase inhibitors), and radiation therapy stress in hepatocellular carcinoma (HCC) and cancer regimens in HCCs and opens the way to the development of new combinatorial therapeutic strategies that will hopefully contribute to HCC eradication and clinical outcomes.

**Materials and Methods**

**Compounds**

Oxaliplatin, chloroquine, 3-methylamphetamine, and N-acetyl-cysteine were purchased from Sigma-Aldrich. Oxaliplatin was dissolved in 100% dimethyl sulfoxide and diluted with Dulbecco’s Modified Eagle’s Media (DMEM) to the desired concentration with a final dimethyl sulfoxide concentration of 0.1% for the in vitro studies. Chloroquine, 3-methylamphetamine, and N-acetyl-cysteine were dissolved in PBS and diluted with DMEM to the desired concentration.

**Cell lines and animals**

The human HCC cell lines Huh7 and SMMC-7721 (18) were routinely maintained in high-glucose DMEM supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 mg/mL streptomycin. Both cell lines were cultured at 37°C in a humidified incubator with an atmosphere of 5% CO2. Male athymic BALB/c nude mice (4 weeks old; Shanghai Institute of Material Medicine, Chinese Academy of Science) were raised under pathogen-free conditions. The animal care and experimental protocols were in accordance with the guidelines established by the Shanghai Medical Experimental Animal Care Commission.

**Patient samples**

Patient samples were obtained after provision of informed consent according to an established protocol approved by the Ethics Committee of Fudan University. Collected data contained no information that could allow identification of the patients. Tumor specimens used in the study were randomly obtained from 40 patients with HCCs who underwent curative resection at the Liver Cancer Institute, Zhongshan Hospital, Fudan University, between 2009 and 2010. Half of the patients underwent locoregional chemotherapy within 3 months before surgery whereas the other half accepted no preoperative treatment. Conventional clinicopathologic parameters were recorded and detailed in Supplementary Table S1. No significant difference was found between HCC samples with and without preoperative chemotherapy. For each patient, the diagnosis of HCC was confirmed by pathologic examination. Normal liver samples were obtained from donors of living liver transplantation.

**Western blot analysis**

Briefly, the proteins from total cell lysates were separated by standard SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were washed, blocked, and incubated with specific primary antihuman antibodies against Atg5, Beclin 1, LC3 (Cell signaling), and GAPDH (Millipore), followed by incubation with horseradish peroxidase–conjugated secondary antibodies. The immunoreactive species were detected using an enhanced chemiluminescence assay.

**Assessment of apoptosis in vitro and in vivo**

Apoptotic cells were evaluated in vitro by Annexin V/propidium iodide staining (Invitrogen) according to the manufacturer’s instructions, as previously described (19). Cells were analyzed via flow cytometry. Data were analyzed using CellQuest software (BD Bioscience). Analysis of DNA
fragmentation using terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) was conducted to assess apoptosis in vivo, using a commercial kit (Oncon) as described previously (20). The number of TUNEL-positive cells was calculated at 200× magnification for 8 fields randomly selected in each tumor sample.

**Autophagy analysis**

Autophagy was assessed using GFP-LC3 redistribution and LC3 mobility shift. Redistribution of GFP-LC3 was detected using an inverted fluorescence microscope. The number of GFP-LC3-positive dots per cell was determined in 3 independent experiments. Eight randomly selected fields representing 200 cells were counted. For the LC3 mobility shift assay, cells were lysed with M-PER (Mammalian Protein Extraction Reagent; Pierce) and then subjected to immunoblot analysis with an antibody against LC3 (Cell Signaling).

**RNA interference**

Two different sequences targeted to 2 different sites in Atg5 were designed without off-target effects. The sense and antisense strands of siRNAs were: 5'-CUGCUATT-3' and 5'-GAAGUUUGUCCUU-UUGGAUA-UUGGAUA-UUGGAUA-AUGCCAUUUCAGTG-3' and 5'-UAGCAGAAGGACAAACUUCTT-3' and 5'-GAAGUUUGUCCUU-UUGGAUA-UUGGAUA-UUGGAUA-AUGCCAUUUCAGTG-3'. Atg5 siRNAs as well as a negative control mismatch sequence were transfected into the HCC cells using Lipofectamine 2000 (Invitrogen). Cells were lysed 72 hours after transfection, and protein (20 μg) was assayed by immunoblot analysis.

**Immunohistochemistry**

Immunohistochemistry was conducted using a 2-step protocol (Novolink Polymer Detection System) as previously described (21). Rabbit anti-human LC3 antibody (Abcam) was used to detect the expression of LC3 in HCC xenografts and human HCC specimens. Under high-power magnification (×200), photographs of 8 representative fields were captured with a Leica QWin Plus v3. The positively stained area of LC3 in each photograph was measured using Image-Pro Plus v6.2 software (Media Cybernetics, Inc.).

**Detection of ROS**

Intracellular ROS was measured in drug-treated and untreated cells as described previously (22). Briefly, cells were washed and resuspended in 500 μL of PBS and were loaded with 10 μmol/L of 2′,7'-dichlorofluorescein diacetate (Invitrogen) for 30 minutes. Cells were then washed and analyzed by flow cytometry. Data were analyzed using CellQuest software (BD Bioscience).

**Electron microscopy**

Cells were immediately fixed with 2.5% glutaraldehyde with 0.1 mol/L sodium cacodylate and stored at 4°C until embedding. Samples were postfixed with 1% osmium tetroxide, followed by dehydration with an increasing concentration gradient of ethanol and propylene oxide. Samples were then embedded and ultrathin (50–60 nm) sections were cut using an ultramicrotome (LKB-I). Images were examined using a JEM-1200 electron microscope at 80 kV after the samples were stained with 3% uranyl acetate and lead citrate.

**In vivo treatment**

HCC tumor models produced by Huh7 were established in nude mice by subcutaneous injection of 5 × 10⁶ Huh7 cells in 0.2 mL of serum-free culture medium into the left upper flank region as described previously (23). Three days later, the mice were randomly assigned to receive 1 of the following 4 treatments: (i) intraperitoneal injection of vehicle twice a week (n = 5); (ii) intraperitoneal injection of chloroquine 60 mg/kg 4 times a week (n = 6); (iii) intraperitoneal injection of oxaliplatin 5 mg/kg twice a week (n = 6); or (iv) a combination of treatments ii + iii (n = 6). Tumor growth was monitored by measuring 2 bisecting diameters of each tumor with a caliper every 5 days from day 15 after implantation. The tumor volume was calculated using the formula (V = a × b²/2), where a represents the largest diameter and b the smallest diameter. Mice were euthanized by day 32 after treatment. Tumors of each group were completely removed, photographed, and fixed in 10% formalin/PBS or stored in liquid nitrogen for histologic examination.

**Toxicity analysis**

To determine whether the combination of chloroquine and oxaliplatin was toxic to animals, the body weight, food consumption, and clinical signs of toxicity (such as ruffled fur, diarrhea, cachexia, or toxic deaths) in the animals were closely monitored. After the mice were sacrificed, blood was collected for analysis of serum alanine aminotransferase and aspartate aminotransferase.

**Statistical analysis**

Means were compared using the unpaired, 2-tailed Student’s t test between 2 groups or by 1-way ANOVA for multiple groups. Categorical data were analyzed using the χ² or Fisher’s exact tests. A value of P < 0.05 was considered significant.

**Results**

**Autophagy is activated in HCC cells after oxaliplatin treatment**

The conversion of the soluble form of LC3 (LC3-I) to the lipidated and autophagosome-associated form (LC3-II) is a
characteristic of autophagy. Thus, we used Western blot analysis to measure the amount of LC3-II in oxaliplatin-treated cells. Increased levels of LC3-II were clearly detected in HCC cell lines after exposure to oxaliplatin (Fig. 1A). In addition, coincubation with the lysosomal protease inhibitor chloroquine, which blocks the final steps of autophagic degradation, enhanced oxaliplatin-induced accumulation of LC3-II (Fig. 1A). We then established Huh7 and SMMC-7721 cells that stably expressed the GFP-LC3 fusion protein. Cells expressing GFP-LC3 showed that the GFP-LC3 signals shifted from a diffuse cytoplasmic pattern to a punctate membrane pattern following 12-hour exposure to oxaliplatin, indicating that autophagic vacuoles are formed (Fig. 1B). Morphometric analysis of the green fluorescent protein (GFP) fluorescence images revealed significantly more GFP-LC3-positive dots per cell in oxaliplatin-treated cells than in untreated cells (Fig. 1C). Consistent with the Western blot data, chloroquine treatment enhanced the dot formation. In contrast, 3-methylamphetamine, which inhibits autophagosome formation, significantly reduced the number of visible dots in the treated cells and redistributed GFP-LC3 to the cytoplasm.

Using a transmission electron microscope, we further analyzed numbers of autophagosome-like vacuoles with double-membrane structures (data not shown). As shown in Figure 1D, morphologic analysis of oxaliplatin-treated Huh7 cells by transmission electron microscopy revealed double-membrane vacuolar structures with the morphologic features of autophagosomes. Taken together, these results indicate that autophagosomes were properly produced, processed, and consumed in these HCC cell lines after oxaliplatin treatment.

**Inhibition of autophagy enhanced cell death after oxaliplatin treatment**

To examine whether the autophagy induced by oxaliplatin has a role in cell survival or cell death, the autophagy inhibitors 3-methylamphetamine and chloroquine were used to examine the cell death of Huh7 and SMMC-7721 cells incubated with oxaliplatin for 24 and 48 hours. Flow cytometric analysis showed that a time-dependent...
induction of cell apoptosis was observed in HCC cells; this was particularly marked in cells treated with the 3-methylamphetamine/oxaliplatin or chloroquine/oxaliplatin combination. The number of Annexin V–positive cells was significantly increased in the 3-methylamphetamine/oxaliplatin- or chloroquine/oxaliplatin–treated cells compared with oxaliplatin-treated cells, especially after 48 hours of exposure (Fig. 2A). However, these inhibitors alone had only a slight effect on the cell death. Similar results were obtained in cells treated with \( \text{atg5} \) siRNAs. To detect the transfection efficacy, a 100 nmol/L concentration of fluorescent oligo was used for the analysis. As seen in Supplementary Figure S1, more than 90% of cells were filled with fluorescent oligo after 4 hours of incubation, which means that the siRNA was successfully transfected into cells. Western blot analysis in Figure 2B also indicated that expression of the Atg5 protein in HCC cells was successfully silenced after siRNA transfection and that Beclin 1 expression was not affected. Atg5 siRNA–transfected cells exhibited a significantly increased vulnerability to oxaliplatin compared with wild-type and negative siRNA-transfected cells. These results indicate that autophagy has a pivotal role in protecting HCC cells against the lethal effects of oxaliplatin.

Oxaliplatin-induced ROS generation is augmented by autophagy inhibition and has an important role in cell death

Oxaliplatin has pleiotropic effects that contribute to its mechanism of action in malignant cells, including the generation of ROS, which have been shown to be a critical event in platinum-induced cell death (22). To determine whether autophagy can modulate oxaliplatin-induced ROS generation, we quantified the intracellular levels of ROS in HCC cells following 12-hour treatment with 3-methylamphetamine, chloroquine, oxaliplatin, or the combination.
In both cell lines, there were marked increases in the generation of ROS in cells treated with 3-methylamphetamine/oxaliplatin or chloroquine/oxaliplatin combinations compared with cells treated with oxaliplatin alone (Fig. 3A). To evaluate whether the enhanced generation of ROS observed in the presence of autophagy inhibitors was an important event underlying its ability to potentiate oxaliplatin-induced cell death, we assayed the ability of the antioxidant N-acetyl-cysteine to abrogate this effect. Huh7 and SMMC-7721 cells pretreated with N-acetyl-cysteine displayed significantly reduced cell death induction in response to 3-methylamphetamine/oxaliplatin or chloroquine/oxaliplatin combinations (Fig. 3B compared with Fig. 2A). Therefore, we concluded that the generation of ROS has an important role in the induction of cell death by oxaliplatin in combination with an autophagy inhibitor.

**Activation of autophagy by oxaliplatin in vivo**

To determine the in vivo relevance of our findings, we first investigated whether oxaliplatin promoted the activation of autophagy in Huh7 cell–derived tumor xenografts. Electron microscopy of these tumors revealed that oxaliplatin induced morphologic changes characteristic of cell death including chromatin condensation, nuclear and cytoplasmic blebbing, and nuclear fragmentation. In live cells, the presence of dense material inside double-membrane–bound vacuoles, which were rarely found in vehicle-treated tumors, indicated that oxaliplatin induced the morphologic characteristics of autophagy in vivo (Fig. 4A). To confirm that these processes occurred in the tumors, we conducted immunostaining of HCC cells to assess the expression levels of LC3 (as a marker of autophagy). Consistent with electron microscopy results, oxaliplatin administration significantly increased the expression of LC3 (Fig. 4B), indicating that LC3 could be used as an autophagy-specific protein in vivo.

**Antimalarial agent chloroquine enhances the anticancer activity of oxaliplatin in vivo**

Chloroquine disrupts lysosomal structure and function, preventing effective autophagic degradation and leading to the accumulation of ineffective autophagosomes (24). We assessed whether treatment with chloroquine would potentiate the effects of oxaliplatin in mice in vivo. Thus, nude mice were implanted with Huh7 tumor xenografts and divided in 4 groups: (i) mice treated with vehicle only, (ii) mice treated with chloroquine only, (iii) mice treated with oxaliplatin only; and (iv) mice treated with chloroquine and oxaliplatin. The tumors were allowed to develop for 35 days after injection, and therapy was started on day 3 after tumor implantation. No differences of body weight, food consumption, and clinical sign of toxicity between vehicle-treated control animals and drug-treated animals were observed. These effects were also achieved without obvious systemic toxicity and damage to liver function (Supplementary Table S2).

At day 35, mice did not show a significant decrease in tumor volume after chloroquine administration compared with the vehicle controls (1,483 ± 318 mm

**Figure 3.** Autophagy augments oxaliplatin-induced ROS generation, which mediates cell death. A, Huh7 and SMMC-7721 cells were treated with 10 μmol/L CQ, 5 mmol/L 3-MA, 10 μmol/L oxaliplatin, or the indicated combinations for 12 hours. Cellular ROS generation was determined using 2,7’-dichlorofluorescein diacetate in conjunction with flow cytometry as described in Materials and Methods. Data shown are mean (SD) from at least 3 independent experiments. *, P < 0.05. B, Huh7 and SMMC-7721 cells were pretreated with 10 mmol/L N-acetyl-cysteine (NAC) for 6 hours. Following pretreatment with NAC, cells were exposed to 10 μmol/L oxaliplatin alone and with 5 mmol/L 3-MA or 10 μmol/L CQ for 48 hours. The percentage of dead cells was determined with Annexin V staining assay. Data shown are mean (SD) from at least 3 independent experiments.
1,592 ± 579 mm³; \( P = 0.701 \). Although the difference was not significant, oxaliplatin treatment induced marked tumor suppression (1,190 ± 170 mm³ vs. 1,592 ± 579 mm³; \( P = 0.14 \)). The oxaliplatin/chloroquine combination was associated with a significant decrease in tumor volume (854 ± 280 mm³) compared with animals treated with either agent alone. As shown in Figure 5A, from day 25, there were significant differences in tumor volume between the oxaliplatin/chloroquine group and oxaliplatin group.

TUNEL staining was conducted on tumor specimens to assess the number of cells that were undergoing cell death. Both oxaliplatin and oxaliplatin/chloroquine treatments resulted in a marked increase in TUNEL-positive tumor cells compared with vehicle-treated tumors (Fig. 5B). Quantification of TUNEL-positive cells per hpf in treated tumors found no significant differences between vehicle- and chloroquine-treated tumors (10.7 ± 2.1 vs. 11.6 ± 3.6; \( P = 0.258 \)). A significantly greater number of TUNEL-positive tumor cells was observed in oxaliplatin/chloroquine-treated tumors than in oxaliplatin-treated tumors (45.8 ± 8.4 vs. 22.5 ± 6.5; \( P < 0.001 \)).

**Autophagy activation in human HCC tissues treated with locoregional chemotherapy**

Evaluating autophagy in clinical tumor samples has been difficult mainly due to the lack of appropriate autophagy-specific markers. Previous work in colon cancer showed that LC3 immunohistochemical staining is a useful surrogate marker for autophagy in surgically resected cancer.
specimens (25). According to our in vivo findings, we evaluated autophagy status in HCC tissue samples with or without locoregional chemotherapy before surgery by detecting LC3. Paraffin-embedded samples of 40 HCC cases (half the cases were HCC with locoregional chemotherapy and half were HCC without preoperative treatment) and 10 normal livers (as a baseline of autophagy in liver) were examined. Immunohistochemical analysis revealed a low expression level of LC3 in tumors without treatment than in normal liver samples. Among these 20 HCCs, 12 samples had lower LC3 levels than baseline and 4 had similar levels. In contrast, LC3 expression was significantly higher after locoregional chemotherapy than in tumors that had not undergone treatment. Among these 20 HCCs, 8 samples had levels higher than baseline and 6 had similar levels (Fig. 6A). To confirm whether LC3 deposition reflects autophagosome formation, HCC specimens with LC3 high expression levels were further examined by transmission electron microscopy; these HCC cells had lipid bilayer structures engulfing organelles in the cytoplasm, which are characteristic of autophagosomes (Fig. 6B).

Discussion

The third-generation platinum drug oxaliplatin has been widely used in cancer treatment including for colon cancer, gastric cancer, and metastatic liver cancer (26). Clinical trials of oxaliplatin in advanced HCCs showed moderate activity but limited efficacy (27–29). Hence, it is important to identify new agents or strategies to kill HCC cells either as single agents or in combination with oxaliplatin. In previous experimental studies, we found that the antitumor effect of oxaliplatin in HCC is due to induction of cell death
Autophagy Promotes Oxaliplatin Resistance in HCC

(23). However, the surviving HCC cells after oxaliplatin treatment underwent epithelial-to-mesenchymal transition and showed increased metastatic potential (30). As a result, we sought to determine the contribution of autophagy, an evolutionarily conserved lysosomal self-digestion process, to the survival of HCC cells during oxaliplatin-induced cell death.

Oxaliplatin treatment of HCC cells and xenografts induced morphologic and biochemical features characteristic of autophagy such as the generation of autophagosomes, GFP-LC3 redistribution, and LC3-II accumulation. More importantly, the autophagic process could be enhanced by the lysosomal protease inhibitor chloroquine and inhibited by 3-methylamphetamine, a nucleotide derivative that inhibits the earliest stages of autophagosome formation, indicating that the autophagic machinery was functionally activated after oxaliplatin treatment. Autophagy is a key mechanism to recycle energy and nutrients during starvation or stress. Although the precise role of autophagy in cell survival versus death is highly context dependent, increasing evidence indicates that autophagy can promote tumor cell survival in response to both cytotoxic and targeted chemotherapies (31, 32). To directly determine the role of autophagy, we assessed whether oxaliplatin-induced cell death was enhanced by chemical and genetic inhibition of autophagy using 3-methylamphetamine and chloroquine, respectively, and RNA interference of the essential autophagy protein LC3. More effectively, but significant, increase in cellular ROS generation upon treatment with either single agent alone. Furthermore, increased levels of ROS generation seem to be required for maximal cell death by these agents because treatment with the antioxidant N-acetyl-cysteine markedly reduced their efficacy.

Drug resistance continues to be a major obstacle that limits the success of cancer therapy, irrespective of tumor type; therefore, the identification and validation of novel therapeutic strategies for chemorefractory disease represent a significant challenge in cancer research (6). The results presented herein showed that the autophagy inhibitor chloroquine in combination with the platinum agent oxaliplatin is associated with cell death and growth inhibition in a relevant preclinical model of human HCCs. The Food and Drug Administration–approved antimalarial agent chloroquine has been used safely for several decades for malaria prophylaxis and may be useful in combination with oxaliplatin for the treatment of advanced or recurrent HCCs. As our study showed that modulation of autophagy is potentially effective in HCC treatment, it will also be interesting to investigate whether autophagy inhibitors can enhance the efficacy of other known or novel pharmacologic antitumor agents.

There are contradictory data on the role of autophagy in oncogenesis and anticancer therapy (38). Although we have previously shown an autophagy defect in human HCC tissues, autophagy status in HCCs after therapeutic stress is largely unknown. In the present work, we showed that the autophagy-specific protein LC3 was significantly accumulated in normal liver samples but not in HCC samples, indicating that autophagy might be suppressed in HCC cells. More importantly, LC3 expression was induced to a significantly higher level in tumors that had been subjected to locoregional chemotherapy than in tumors that had not been treated. Accumulated LC3 protein has been proposed to be involved in autophagosome formation. The subcellular distribution of LC3 was similar to that of autophagosome-incorporated LC3 in culture cells. In addition, the ultrastructure revealed the presence of autophagosomes in the cytoplasm. Therefore, we speculate that the autophagy is activated in human HCCs under therapy stress such as oxaliplatin treatment.

Overall, we conclude that autophagy activation under therapy stress contributes to HCC tumor cell survival both in vitro and in vivo. Autophagy inhibition, using chemical and genetic inhibition of autophagy, potentiates oxaliplatin cytotoxicity against HCC cells by increasing the level of ROS generation. On the basis of these results, we predict that targeting the autophagy pathway is a promising therapeutic strategy to enhance the effects of chemotherapy and improve clinical outcomes in HCC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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