Antitumor activity of integrin $\alpha_V\beta_3$ antibody conjugated-cationic microbubbles in liver cancer

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**Background:** The overexpression of integrin $\alpha_V\beta_3$ in hepatocarcinoma (HCC) promotes tumor progression, metastasis, and clinical staging. Thus, the inhibition of integrin $\alpha_V\beta_3$ might be potentially effective as an anti-cancer agent in HCC.

**Methods:** In this study, we aimed to investigate the antitumor effect of integrin $\alpha_V\beta_3$ antibody conjugated cationic microbubbles (CMBs) in HCC model. By conjugating with integrin $\alpha_V\beta_3$ antibody with non-targeting CMBs, CMBs$_{\alpha_V\beta_3}$ was constructed. The antitumor effect of CMBs$_{\alpha_V\beta_3}$ was evaluated in HepG2 cells in vitro and in HepG2 xenograft mouse models. Bcl-2, p53 and CD31 mRNA level, and caspase-3 activity were examined in xenograft tumors. Cell proliferation assay and scratch test were performed to evaluate the anti-migrant effect of CMBs$_{\alpha_V\beta_3}$ in vitro.

**Results:** CMBs$_{\alpha_V\beta_3}$ could specifically target to HCC HepG2 cells and improve pEGFP-KDRP-CD/TK plasmid transfection efficiency. In HepG2 xenograft mice models, CMBs$_{\alpha_V\beta_3}$ treatment significantly suppressed tumor weights and volumes. CMBs$_{\alpha_V\beta_3}$ treatment suppressed Bcl-2 and p53 mRNA level in tumors. In HepG2 cells, CMBs$_{\alpha_V\beta_3}$ significantly impaired wound healing and inhibited cell proliferation. Moreover, when combined with CD/TK double suicide gene transfection and 5-FC/GCV treatment, caspase-3 was activated and the cell proliferation was tremendously inhibited.

**Conclusions:** CMBs$_{\alpha_V\beta_3}$ not only suppresses cell migration and proliferation, but also facilitates 5-FC/GCV plus CD/TK double suicide gene-induced apoptotic cell death. CMBs$_{\alpha_V\beta_3}$ is a promising gene delivery agent with potential anti-tumor activity itself.

**Keywords:** Integrin $\alpha_V\beta_3$ antibody; conjugated-cationic microbubbles (CMBs); anti-tumor activity; HepG2 xenografts; migration

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**Introduction**

Integrins are cell-surface glycoproteins that trigger a diversity of signaling pathways, including cell adhesion and angiogenesis (1,2), and are involved in various human cancers. Integrin expression is hypothesized to render cancer cells to proliferate and migrate (3). Thus, integrins represent attractive targets for the prevention of cancer spread and tumor progression (4). Integrin $\alpha_V\beta_3$ is constitutively expressed in quiescent endothelial cells at low level (5), but it is highly expressed in tumors, such as prostate cancer (6), breast cancer (7), and melanoma (4).
Integrin αvβ3 overexpression in hepatocarcinoma (HCC) is associated with PI3K/Akt and TGF-β/ERK signaling pathways and promotes tumor progression, metastasis, and clinical staging (8-10).

The inhibition of integrin αvβ3 might suppress tumor proliferation. A series of integrin αvβ3 inhibitors have been developed to target tumors. RGD (Arg-Gly-Asp) peptides, a group of canonical antagonist of integrin αvβ3, show inhibitory activity in cell mobility and cell attachment in breast cancer (11). By enhancing internalization rate of these micelles in melanoma, the integrin αvβ3 targeting peptide (RGDFK) shows synergistic cytotoxicity with docetaxel/cisplatin-coated micelles (12). Besides RGD peptides, by binding to NC1 domain of collagen XIX, integrin αvβ3 could inhibit FAK/PI3K/Akt/mTOR pathway (13).

Our previous study also has demonstrated that by conjugating with αvβ3 integrin antibody, non-targeting cationic microbubbles (CMBs) can specifically target to HepG2 cells (14), and substantially increase pEGFP-KDRP-CD/TK plasmid transfection efficiency. The development of ultrasound contrast agent opens up a new idea of carrying target-delivery genes or drugs for chemotherapy in liver tumor patients (15). Ultrasound-targeted microbubble destruction (UTMD) provides a non-invasive, safe, and repeatable method for gene delivery (16-18). To further improve gene delivery efficiency, researchers design conjugated MBs to specifically bind to the membrane proteins on the surface of tumor cells. In the present study, CMBsαvβ3 could significantly suppress tumor growth in HepG2 xenografts mice model. In vitro, CMBsαvβ3 significantly impaired the wound healing and inhibited cell proliferation of HepG2 cells. CMBsαvβ3 also facilitated 5-FC/GCV + CD/TK double suicide gene-induced anti-tumor activity. These findings suggested CMBsαvβ3 as a promising gene delivery agent with potential anti-tumor activity itself.

Methods

Cell line and preparation of non-targeting CMBs

As previously described (14), human liver cancer HepG2 cells were purchased from Xiangya Cell Bank, Central South University (Changsha, China). 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), dipalmitoylphosphatidylcholine (DPPC), biotinylated dipalmitoylphosphatidylyl-ethanolamine (DSPE-PEG2000-Biotin) and 1,2-distearyl-3-trimethyl-ammonium-propane (DTAP; Avanti, Alabaster, AL, USA) were mixed in a 5 mL plastic tube to form a suspension at a molar ratio of 46:36:8:2 (19). Perfluorinated propane (C₈F₁₈) was purchased from the Special gas Co., Ltd. Factory (Nanjing, China). Following lyophilization, 1 mL of phosphate-buffered saline (PBS) was added to the samples to rehydrate them and then C₈F₁₈ gas was slowly injected into the container to replace the air. Samples were then agitated using an ultrasonic mechanical vibrator with high speed shearing method for 90 s to form a milky white solution.

Preparation of CMBsαvβ3

Integrin αvβ3 antibody was conjugated to the distal end of the DSPE-PEG2000-Biotin molecules through biotin-streptavidin coupling chemical method (20). Briefly, 500 μL CMBs (1×10⁷/mL) were mixed with 100 μg biotinylated anti-αvβ3 antibody in an ultrasonic agitating reaction for 30 min. Then, after centrifugation at 50 g for 5 min, the upper layer (CMBsαvβ3) was washed with PBS three times and then collected. The morphology and particle distribution of CMBsαvβ3 was observed by optical microscopy. The particle size and surface potential were measured by a Zetasizer 3000HS (Malvern, Worcestershire, UK). All experiments were performed for five times.

Plasmids

As described in our previous study (14), the restructured plasmid pEGFP-KDRP-CD/TK coding for green fluorescent protein (GFP) contained CD/TK double suicide gene and was driven by KDR promoter (21). The molecular weight of this plasmid is 2,300 kDa with 3,486 bp. The plasmid was amplified and then isolated and purified using QIAGEN plasmid giga kit (Qiagen, Valencia, CA, USA) following the manufacturer’s protocol.

Fluorescent assay

To confirm binding of CMBsαvβ3 to HepG2 cells, rhodamine mouse anti-human immunoglobulin (Ig) G was adopted to detected αvβ3 antibody. After treated with CMBsαvβ3 alone or plus pEGFP-KDRP-CD/TK plasmid, HepG2 cells were incubated with rhodamine mouse anti-human Ig G for 1 h at room temperature. Unbound (Ig) G was removed by two rounds of centrifugal washing. The binding was observed under a fluorescence microscope (CKX41; Olympus, Tokyo, Japan). EGFP expression levels were also evaluated by fluorescence microscope.
Animal experiments

As described previously (14), nude mice bearing HepG2 liver cancer were randomly divided into 3 groups: normal saline (NS) group, CMBs treatment group and CMBsαβ3 treatment group. All treatments were terminated at 10 days. The tumor growth was observed and tumor volumes were calculated. Five days after treatment, HepG2 xenografts were removed and weighted. Tumor tissues RNA were extracted for quantitative RT-PCR (qRT-PCR) and caspase-3 activity assay.

qRT-PCR screening

Tumor RNA was isolated and converted into cDNA. qRT-PCR was performed using ABI-7500 (AppliedBiosystems, Fostercity, CA, USA) by mixing equal amounts of cDNAs, iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and specific primers. All real-time data were normalized to β-actin.

Caspase-3 activity assay

Tumor tissue protein was extracted as previously described (22). Caspase-3 activity was tested instantly using the colorimetric substrate Ac-DEVD-pNA (Beyotime, Nanjing, China) according to the manufacturer's instructions. After incubation overnight at 37 °C, caspase-3 activity was measured on an enzyme-linked immunosorbent assay instrument (Bio-Rad, USA) at 490 nm.

Scratch closure test

The migration of HepG2 cells exposed to CMBsαβ3 was evaluated by scratch closure test. We took images of the scratched area at 0, 12, and 24 h after scratch and measured widths of the scratched by the Image-Pro Plus 6.0 software.

Anti-tumor effect evaluation

To determine the effects of CMBsαβ3 on the cell cycle, propidium iodide (PI) staining after 75% alcohol fixation was used, followed by flow cytometry analysis. MTT assay was performed to evaluate the cell proliferation inhibition after CMBsαβ3 treatment.

Statistical analysis

All data are expressed as mean ± standard deviation (SD), unless otherwise noted. Differences among groups were analyzed by one-way ANOVA and LSD method for multiple comparisons among groups using the SPSS software package (Version 19.0 for windows, SPSS, Chicago, Illinois, USA) with P<0.05 considered statistically significant.

Results

CMBsαβ3 treatment suppresses HepG2 tumor growth

To investigate the anti-tumor effect of CMBsαβ3 in vivo, HepG2 xenograft mice models were used. The nude mice bearing HepG2 liver cancers were randomly divided into 3 groups: NS group, CMBs treatment group and CMBsαβ3 treatment group. The whole treatment period was 10 days. Tumor volumes were evaluated at day 0 to day 17 from the treatment initial day. As shown in Figure 1, mice in NS (Figure 1A), CMBs (Figure 1B), CMBsαβ3 (Figure 1C) groups were sacrificed and tumors were removed and weighed at 17 days after treatment. Compared to control (NS) group, CMBsαβ3 treatment significantly suppressed tumor growth (P<0.05; Figure 2A). But CMBs treatment had no such effect. The xenograft tumors were weighed at the experiment endpoint. Compared to control group and CMBs group, CMBsαβ3 treatment significantly suppressed tumor weight (P<0.05, Figure 2B). Mice body weights of all three treatment groups did not change significantly during the experiment period (Figure 2C). These findings implied an anti-tumor effect of CMBsαβ3.

CMBsαβ3 binds to tumor cells in xenografts and suppresses Bcl-2 and p53 mRNA level

CMBsαβ3 is supposed to specifically bind to integrin αvβ3 on HepG2 cell surface through the conjugated integrin αvβ3 antibody. To confirm this binding, we adopted rhodamine mouse anti-human immunoglobulin (Ig) G and fluorescence microscope to detect the localization of CMBsαβ3 on HepG2 cells. As demonstrated in Figure S1, the binding of CMBsαβ3 to the surface of HepG2 cells was detected under fluorescence microscope. The red fluorescent signals suggested a tight binding of CMBsαβ3 to HepG2 xenograft cells.

In previous reports, integrin αvβ3 inhibitor has shown effect to inhibit Bcl-2 expression (23) and modify p53 level (24). Consistent with these reports, qRT-PCR results in Figure 3A,B showed that CMBsαβ3 treatment significantly suppressed the Bcl-2 and p53 mRNA levels, compared to control. CD31 mRNA level was not affected by CMBsαβ3.
treatment (Figure 3C). No obvious caspase-3 activation was detected in CMBs\textsubscript{αvβ3} treatment group (Figure 3D). These data implied that the suppression of Bcl-2 and p53 might be responsible for CMBs\textsubscript{αvβ3}-mediated anti-tumor effect. But caspase-3-mediated apoptosis pathway was not involved in CMBs\textsubscript{αvβ3}-mediated anti-tumor effect.

**CMBs\textsubscript{αvβ3} inhibits HepG2 cells proliferation and migration in vitro**

The anti-tumor CMBs\textsubscript{αvβ3} effect of was also evaluated by cell cycle and MTT assays. As shown in Figure 4A, cell cycle distribution was not modified by CMBs\textsubscript{αvβ3} treatment. MTT assay showed that HepG2 cell proliferation was obviously inhibited by CMBs\textsubscript{αvβ3} treatment (Figure 4B). Integrin α\textsubscript{v}β\textsubscript{3} triggers signaling pathway to promote cell adhesion and migration (3). To examine the anti-migrant effect of CMBs\textsubscript{αvβ3}, HepG2 cells were exposed to CMBs\textsubscript{αvβ3} for 24 h, and then evaluated by scratch closure test. At 24 h after scratch, areas of the scratched wounds were measured by Image J software (Figure 5). Compared to NS treatment (Figure 5A), at 24 h after scratch, CMBs\textsubscript{αvβ3} treatment significantly inhibited the healing of scratched wounds (Figure 5B).

**CMBs\textsubscript{αvβ3} facilitates CD/TK transfection and promotes antitumor activity**

In our previous publication (14), we had demonstrated that CMBs\textsubscript{αvβ3} combined with CD/TK transfection + 5-FC/GCV had a higher suppressing effect in HepG2 xenograft mouse model than CD/TK transfection + 5-FC/GCV alone. Moreover, CMBs\textsubscript{αvβ3} plus CD/TK + GCV/5-FC treatment induced more TUNEL-positive cells than CD/TK transfection + 5-FC/GCV treatment alone (14). Here, as demonstrated in Figure S1, the green fluorescence signals indicated a successful CD/TK transfection in HepG2 cells. qRT-PCR assay further confirmed the expression of CD and TK mRNA in HepG2 cells (Figure S2). When coupled with CMBs\textsubscript{αvβ3}, CD/TK gene expression levels were higher.
Figure 2 CMBs\(_{α\text{v}β3}\) inhibited tumor growth in HepG2-bearing nude mice. Differences among groups were analyzed by one-way ANOVA and LSD method for multiple comparisons among groups with P < 0.05 considered statistically significant. (A) Tumor volumes were measured on day 0 to day 27 for every three days. The tumor volume in CMBs\(_{α\text{v}β3}\) group was significantly lower compared with NS group; (B) the tumor weight in CMBs\(_{α\text{v}β3}\) group was significantly lower compared with CMBs group and NS group; (C) mice body weights kept unchanged among all treatment groups. *, P < 0.05. NS, normal saline; CMBs, cationic microbubbles.

Figure 3 CMBs\(_{α\text{v}β3}\) suppresses Bcl-2 and p53 mRNA level. Differences among groups were analyzed by one-way ANOVA and LSD method for multiple comparisons among groups with P < 0.05 considered statistically significant. mRNA expression levels of Bcl-2 (A), p53 (B) and CD31 (C) were determined by qRT-PCR. β-Actin was used as a control to confirm equal loading of cDNAs. Data are shown as means ± SD of three experiments; (D) caspase-3 activity was evaluated colorimetric substrate and measured by Bio-Rad reader. Data are shown as means ± SD of three experiments. *, P < 0.05. NS, normal saline; CMBs, cationic microbubbles; qRT-PCR, quantitative RT-PCR; SD, standard deviation.
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α 72 h. These data implied that caspase-3-mediated
Figure 5E
α 48 h α
α

healing (P<0.05). Moreover, compared to CD/TK +
treatment alone could significantly suppress the wounds
both CMBs
+ GCV/5-FC could significantly suppress the healing of
wound healing. When combined with CMBs
TK suicide gene transfection alone did not prevent the
was also evaluated. As demonstrated in
Figure S2B,C
standard deviation.

Cell inhibition (%)

10 20 30 40 60 70

0 10 20 30 40 50 60 70 80 90

Figure 4 CMBs
α
v
β
3

inhibits cell proliferation in vitro. Differences
among groups were analyzed by one-way ANOVA and LSD
method for multiple comparisons among groups with P<0.05
considered statistically significant. (A) The effects of CMBs
α
v
β
3

Figure 5C,
comparing the
Figure 5F

CD/TK suicide gene transfection and promoting 5-FC/
integrin-overexpressed tumor cells; (II) facilitating
anti-migrant effect of CD/TK + GCV/5-FC treatment.

inhibited HCC cell migration itself, but also increased the
rate (P<0.05). These results implied that CMBs
α
v
β
3
TK + GCV/5-FC treatment had even lower wound healing
rate (P<0.05). These results implied that CMBs
α
v
β
3
not only inhibited HCC cell migration itself, but also increased the
anti-migrant effect of CD/TK + GCV/5-FC treatment.
While no obvious caspase-3 activation was detected in
CMBs
α
v
β
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+ GCV/5-FC treatment group, CD/TK + 5-FC/GCV treatment
and CD/TK + 5-FC/GCV plus CMBs
α
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treatment could induce significant caspase-3 activation in HepG2 cells
(Figure 6A). These data implied that caspase-3-mediated
apoptosis pathway was involved in CD/TK + 5-FC/GCV-
mediated anti-tumor effect, but not in CMBs
α
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treatment. MTT assay showed a significant higher suppression in
cell proliferation in CD/TK + 5-FC/GCV plus CMBs
α
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treatment group, compared to CMBs
α
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alone or CD/TK +
5-FC/GCV treatment alone group (Figure 6B). Based on
these findings, we suggested that CMBs
α
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not only had potential anti-tumor activity itself, but also facilitated CD/
TK gene transfection and promoted the anti-tumor effects
(Figure 7).

Discussion

In the era of precision medicine, targeting and accuracy
become more and more important and practicable.
Application of CMBs to treat cancers (25), brain disease
(26,27), hepatic fibrosis (21), and heart diseases (28) are
non-invasive, targeted and safe. Our designed CMBs
α
v
β
3

include following two aspects: (I) inhibiting migration of
integrin
α
v
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3
on HepG2 surface, and showed
high affinity to integrin
α
v
β
3
on HepG2 surface; and showed
potential anti-tumor activity in HepG2 mice models. This
integrin
α
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3
targeting system not only suppressed tumor
growth alone, but also promoted CD/TK gene expression
and 5-FC/GCV killing effect. Unlike other integrin ligands/
analogues/peptides or integrin-based drug-load systems,
CMBs
α
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initiated a completely new research field for the
combination of multiple anti-tumor effects in one system.
We believe the major anti-tumor actions of CMBs
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includes following two aspects: (I) inhibiting migration of
integrin
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-overexpressed tumor cells; (II) facilitating
CD/TK suicide gene transfection and promoting 5-FC/
GCV-induced apoptotic cell death. This proposed model
was described in Figure 7.
Biotinylated specific antibodies coupled to streptavidin-
containing microbubbles through the biotin-streptavidin
linkage have been used for molecular ultrasound imaging
to monitor the receptor expression (29), such as VEGF receptor (30,31). Like Cyclic RGD peptides, biotin-streptavidin linkage is commonly used to specifically couple quantum dots to integrins (32). Cilengitide, a cyclic pentapeptide, is efficient to treat glioblastoma by targeting integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (33,34). Combination of UTMD with cilengitide-nanotherapy increases the tumor Cilengitide level over 3-fold and significantly reduce renal clearance, which help reduce Cilengitide dose level and increase killing effect (35). From our animal experiment results, focused UTMD with CMBs$\alpha_v\beta_3$ showed significantly anti-tumor effect in HepG2 xenograft tumors (Figure 1).

Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ ligands contain the RGD sequence. Cyclic RGD peptides are canonical integrin inhibitors, such as cilengitide (36). Humanized monoclonal integrin antibodies abituzumab and vedolizumab have demonstrated anti-tumor activity preclinically (37). Other inhibitors including salvicine and borrelidin are non-specific (38). CMBs$\alpha_v\beta_3$ specifically targets to integrin $\alpha_v\beta_3$ and showed high affinity to HepG2 cells in our models.

Figure 5 CMBs$\alpha_v\beta_3$ inhibits migration. Differences among groups were analyzed by one-way ANOVA and LSD method for multiple comparisons among groups with P<0.05 considered statistically significant. Effects of NS (A), CMBs$\alpha_v\beta_3$ (B), CMBs (C), CD/TK transfection + GCV/5FC (D) and CMBs$\alpha_v\beta_3$ + CD/TK transfection + GCV/5FC (E) on wound closure in HepG2 cells. 10 visions were chosen at random and each experiment was repeated for three times (mean ± SD of three experiments); (F) wounds healing areas were compared among five treatment groups. CMBs$\alpha_v\beta_3$ and CD/TK transfection + GCV/5FC treatment significantly suppressed wounds healing. Compared to CD/TK transfection + GCV/5FC treatment, CMBs$\alpha_v\beta_3$ + CD/TK transfection + GCV/5FC further inhibited wounds healing. Data are shown as means ± SD of three experiments. *, P<0.05; **, P<0.01. NS, normal saline; CMBs, cationic microbubbles; SD, standard deviation.
Integrin inhibitors always demonstrate anti-angiogenic (39) and anti-metastasis (40) functions. By targeting to integrin $\alpha \text{V} \beta 3$ on neovasculature, cilengitide increased systemic radio-immunotherapy efficacy of therapy in p53 mutant and bcl-2 overexpressing breast cancer cells (41). In this study, CMBs $\alpha \text{V} \beta 3$ significantly suppressed Bcl-2 and p53 levels, while CD31 level was moderately suppressed in mice models (Figure 3). In vitro experiment and inhibited HepG2 cell migration (Figure 5). Caspase-3 activity was not influenced by CMBs $\alpha \text{V} \beta 3$, which is consistent with our previous study that CMBs $\alpha \text{V} \beta 3$ alone did not induce apoptotic death in HepG2 animal model (14).

Conclusions

CMBs $\alpha \text{V} \beta 3$ exerted the anti-tumor activities by inhibiting migration of integrin $\alpha \text{V} \beta 3$-overexpressed tumor cells and facilitating CD/TK suicide gene transfection and promoting 5-FC/GCV-induced apoptotic cell death. Application of CMBs $\alpha \text{V} \beta 3$ could initiate an accurate-targeting field for the combination of multiple anti-tumor effects in one system.

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(Figure S1).

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.05.29). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The animal experiment in this study complied with the Third Xiangya Hospital guidelines and approved by the Third Xiangya Hospital Ethics Committee (2012-S119).

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**Figure S1** Binding of CMBs to HepG2 cells in xenograft tumors. (A) Expression of CD/TK in tumor cells was detected by EGFP green fluorescence. Red fluorescence was observed on the surface of CMBs using immunofluorescent microscopy. Ten visions were chosen at random and each experiment was repeated for three times (mean ± SD of three experiments); (B) CMBs specifically targets to integrin αvβ3 and showed high affinity to HepG2 cells in our models. CMBs, cationic microbubbles; SD, standard deviation.

**Figure S2** CMBsαβ3 facilitates CD/TK gene transfection in HepG2 cells. (A) RT-PCR showed the expression level of CD and TK mRNA in CMBsαβ3 + CD/TK group was higher than that in CD/TK group; mRNA expression levels of CD (B) and TK (C) were determined by qRT-PCR. β-Actin was used as a control to confirm equal loading of cDNAs. Data are shown as means ± SD of three experiments. *, P<0.05; **, P<0.01. NS, normal saline; CMBs, cationic microbubbles; qRT-PCR, quantitative RT-PCR.