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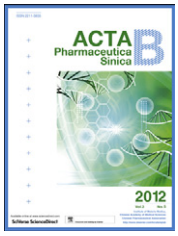

ORIGINAL ARTICLE

Salmonella typhimurium strain SL7207 induces apoptosis and inhibits the growth of HepG2 hepatoma cells *in vitro* and *in vivo*

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KEY WORDS

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Abstract *Salmonella typhimurium* is probably most extensively studied tumor-targeting bacteria and SL7207 is one of its attenuated strains. SL7207 was first made for bacterial vaccine development and its therapeutic efficacy and safety for hepatocellular carcinoma has not been characterized. In this study, the inhibitory ability of SL7207-*lux* on human hepatoma HepG2 cells was tested *in vitro* and *in vivo*. A bacterial luminescent gene cluster (*lux* CDABE) was transfected into SL7207 to better monitor the invasion of the bacteria. The results show that SL7207-*lux* can rapidly enter HepG2 cells and localize in the cytoplasm. This invasion represses cell proliferation and induces apoptosis. *In vivo* real-time invasion studies showed that the bacteria gradually accumulate in the tumor. This enrichment was confirmed by anatomic observation at 5 days after inoculation. About 40% of tumor growth was inhibited by SL7207-*lux* at 34 days post-treatment without significant loss of body weight. The area of necrosis of tumor tissue was clearly increased in the treated group. Bacterial quantification showed that the number of colony-forming units per gram of bacteria within tumor tissue was approximately 1000-fold higher than that of liver and spleen. These data suggest that attenuated *S. typhimurium* strain SL7207 has potential for the treatment of cancers.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies, accounting for more than 748,300 new cases per year worldwide. It is the third and second highest cause of global and Chinese cancer-related deaths, respectively[1]. Although chemotherapy, radiotherapy and transplantation are used in the clinic, the mortality of hepatoma still remains high because of rapid tumor progression, recurrence, and drug-resistance. Though other therapeutic options such as percutaneous ethanol injection, microwave therapy, cryotherapy and transarterial chemoembolization are available, the therapeutic efficacy of these treatments is limited. Development of novel therapeutic strategies targeting hepatocellular carcinoma is thus of high priority[2].

Using bacteria like *Salmonella enterica serovar typhimurium* as alternative cancer therapeutics has sporadically been explored for some time. Salmonella can invade many different host cells, but preferentially colonize solid tumors at tumor-to-liver ratios of 250:1–9000:1, and usually results in tumor growth retardation[3]. These bacteria also accumulate in metastases after systemic administration[4]. Attenuated *S. typhimurium* strains were constructed to reduce the toxicity while retaining the tumor therapeutic effects. The genetically-modified *S. typhimurium* strain VNP20009 has shown promising tumor therapeutic potential but failed in a phase I clinical trial, although it could be safely administered to patients[5]. Another widely used genetically-modified *S. typhimurium,* strain SL7207, is attenuated by deleting aroA, which is critical for aromatic amino acid synthesis[6]. This mutation led to the growth limitation of SL7207 within the host cells because mammalian cells do not synthesize aromatic amino acids. SL7207 had been used as a bacteria vaccine and also showed promising therapeutic potential for breast[7], colon[8], lung[9], prostate cancer[10], neuroblastoma[11] and fibrosarcoma[12] in pre-clinical experiments. Its usage in hepatic carcinoma is still unknown.

In this study, attenuated *S. typhimurium* strain SL7207 was labeled with luciferase to make it easily monitored with a bioluminescence imaging system. Human hepatoma HepG2 cells were used to test the anti-tumor efficacy of SL7207-*lux* both *in vitro* and *in vivo*. We propose that SL7207-*lux* may safely and effectively inhibit hepatoma tumor growth and may be a candidate for development as a therapeutic anticancer agent.

2. Materials and methods

2.1. Bacterial culture

Attenuated *S. typhimurium* strain SL7207 (*S. typhimurium* 2337-65 derivative hisG46, DEL407 [aroA::Tn10 {Tc-s}]), was a kind gift from Dr. Bruce Stocker.

For strain manipulations and maintenance, bacteria were grown at 37 °C in Luria–Bertani (LB) medium and on LB agar plates using standard procedures. In order to determine the bacterial count, 100 μL of culture was serially diluted and plated on LB agar. Bacterial colonies were counted after overnight incubation at 37 °C. The final calculation was based on 1 OD unit of bacteria medium contained 10^9 bacteria.

2.2. Plasmids and transformation

In order to better monitor colonization of the attenuated *S. typhimurium* strain SL7207, we first transformed these cells to express a bacterial luminescent gene cluster (*lux*CDABE). The plasmid (pXen-18) containing *lux* gene operon was bought from Xenogen (USA). Electroporation was used to transform the *lux* gene plasmid operon into the attenuated *S. typhimurium* strain SL7207. 100 μL of frozen competent cells were mixed with 1 μL DNA and treated at 2.5 kV, 25 μF, and 200 Ω in an electroporator (Bio-rad, Gene pulser Xcell System). After electroporation, the bacteria were transferred and cultured in 1 mL of LB medium for 1 h at 37 °C. Transformed culture (200 μL) was then plated onto LB plates with 100 μg/mL ampicillin and cultured overnight at 37 °C.

2.3. Bioluminescence test for monitoring attenuated *S. typhimurium*

Attenuated *S. typhimurium* SL7207-*lux* cells were cultured in LB medium at 37 °C with 100 μg/mL ampicillin overnight. For *in vitro* detection different amounts of cells were plated in a blacken 96 well (Costar) and total photon emission from each sample was captured and quantified using an IVIS 200 Imaging system (Xenogen Corp.).

2.4. Measurement of bacteria invasion

Human hepatoma HepG2 cells were cultured in MEM medium supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. For bacterial invasion, HepG2 cells were plated in 24-well plates and cultured for 24 h. Before treatment the HepG2 cells were rinsed 3 times with MEM without serum and antibiotic. Bacteria in log-phase growth were diluted in cell culture medium MEM without serum and antibiotic and added at the desired multiplicity of infection (MOI) of 1:25, 1:225, 1:675 and 1:2025. After being exposed to bacterial at 37 °C for 1 h, cells were washed three times with MEM without serum and antibiotic and replenished with fresh complete medium containing 100 μg/mL of gentamicin sulfate for 1.5 h to kill extracellular but not intracellular bacteria. The cells were then incubated with complete medium containing 10 μg/mL of gentamicin sulfate for another 3.5 h. At this time, cells were washed three times with PBS and intracellular bioluminescence was observed under an IVIS 200 Imaging System directly[13]. Adherent bacteria could be released by incubation with 0.2 mL 0.1% Triton X-100 for 10 min. LB broth (0.8 mL) was then added and each sample was vigorously mixed.

Adherent bacteria were quantified by plating in order to count colony-forming units (CFU) on LB agar medium. Intracellular bacteria also could be shown using immunofluorescence staining using an anti-*S. Typhimurium* 0–4 antibody [1E6] (abcam).

2.5. Cell proliferation assay by SRB

Human hepatoma HepG2 cells were seeded in 24-well plates at a density of 10^4 cells/well and then treated with different amounts of attenuated *S. typhimurium* SL7207. After 24, 48, or 96 h, cells were assayed by SRB as previous described[14]. The optical density is determined with a micro-culture plate reader (BioTek Elx800) at 570 nm. The percentage of cell survival was calculated.

2.6. Apoptosis assay

Human hepatoma HepG2 cells were seeded in 24-well plates at a density of 2×10^4, then treated with quantified amounts of attenuated *S. typhimurium* SL7207. After 48 h, cells were stained by the DeadEnd^{TM} Fluorometric TUNEL System Kit (Promega, Madison, Wisconsin, USA) following the manufacturer's instruction.

To quantify apoptosis, caspase-3 activity was assayed by Caspase-Glo assay kit (Promega, Madison, USA) according to the manufacturer's protocol. Caspase-3 activity was expressed as the fold increase as compared to untreated cells. The experiments were repeated three times with duplicate slides for each condition.

2.7. In vivo tumor model

Mice used in this study were purchased from the Institute for Experimental Animals, Chinese Academy of Medical Sciences & Peking Union Medical College. The study protocols were in accordance with the regulations of Good Laboratory Practice for non-clinical laboratory studies of drugs issued by the National Scientific and Technologic Committee of People's Republic of China.

Female BALB/c nude mice (18–22 g) were implanted by subcutaneous injection of 5×10^6 human hepatoma HepG2 cells on the right flank. After 3 weeks, the tumors were aseptically dissected and pieces of tumor tissue (2 mm^3 in size) were transplanted *s.c.* by a trocar into mice. When the tumor size was over 100 mm^3, mice were divided into groups ($n=6$) and injected intravenously with saline or 5×10^6 attenuated *S. typhimurium* SL7207-*lux* twice a week for 5 weeks. Tumor size was measured every 3 days and tumor volume was determined by length $\times$ width^2/2. Every week after injection the animals were placed on a warmed stage inside the camera box (IVIS 200 Imaging System, Xenogen) to observe tumor growth. At day 34, tumors were taken from the mice and weighed. The tumor growth inhibition (TGI) for SL7207 treatment group was calculated by the fomula: [1–tumor weight (treated)/tumor weight (control)] $\times$ 100%. In addition, tumor tissues were taken from nude mice on day 34 for hematoxylin–eosin staining after formalin–fixation and paraffin-embedding. Bacteria inside the tissue were released and quantified as after homogenization of the tissue with a DY89-II tissue grinder (Xinzhi Biotechnology Comp., Ningbo, China).

2.8. Statistics

Data were described as means $\pm$ SD of the indicated number of separate experiments. A one-way analysis of variance was performed for multiple comparisons. If there was significant variation between treatment and control groups, the mean values were compared using Student's *t*-test. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Attenuated S. typhimurium SL7207-lux invaded HepG2 cells and located in the cytoplasm

In order to better detect the location and investigate the invasion process of the attenuated *S. typhimurium* strain SL7207, we first transformed these cells to express a bacterial luminescent gene cluster (*lux*CDABE). Briefl, a plasmid pXen-18 containing the *lux* gene operon was transfected into attenuated *S. typhimurium* SL7207. The resulting attenuated SL7207-*lux* cells could express the bioluminescence genes and produce light stably at 37 °C without exogenous substrate supplied. After seeding bacteria in a blacken plate bioluminescence was measured with an IVIS 200 Imaging System. Bioluminescence was positively correlated to the number of cells. The minimum detectable number of bacteria was 3.75×10^6 bacteria *per* well (Fig. 1).

To measure *in vitro* cell invasion efficiency of SL7207-*lux*, bacteria in logarithmic growth phase (Fig. 2A) were seeded onto the plates of hepatoma HepG2 cells and co-cultured for 3.5 h. Intracellular bioluminescence was observed under an IVIS 200 Imaging System directly. The results showed that intracellular bioluminescence was positively correlated to the number of bacteria added to the medium of the HepG2 cells

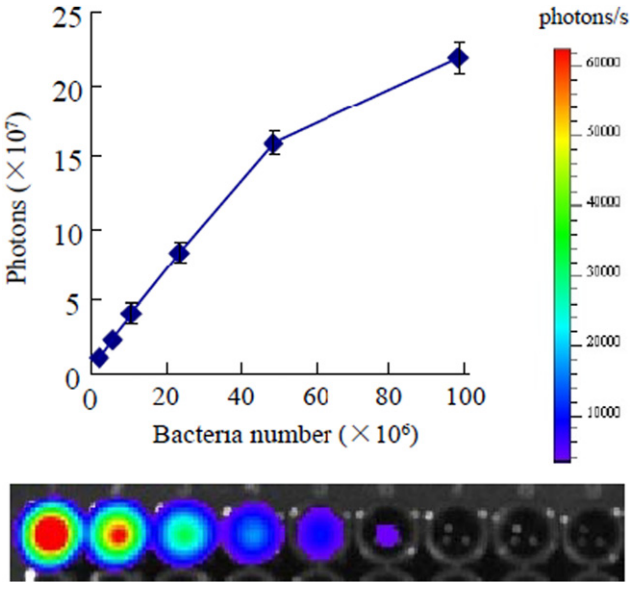

Figure 1 Bioluminescence of attenuated *S. typhimurium* SL7207-*lux*. Bacteria were diluted in 100 μL LB medium and seeded into a blacken 96-well-plates. The plate was imaged using an IVIS 200 imaging system.

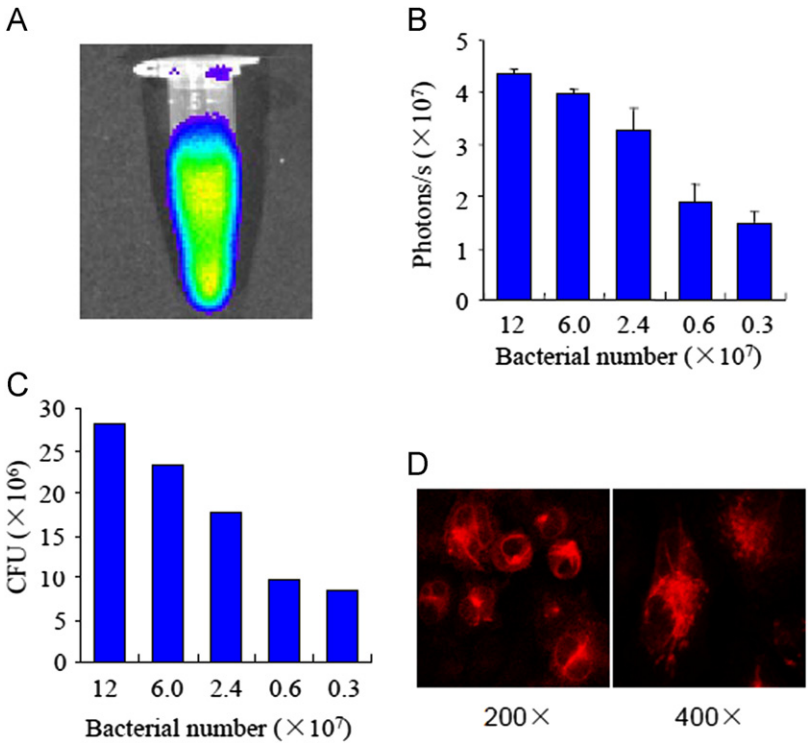

Figure 2 Attenuated *S. typhimurium* SL7207-*lux* invaded HepG2 cells and located in the cytoplasm. SL7207-*lux* bacteria (A) were seeded with HepG2 and co-incubated for 3.5 h, after which luminescence was measured (B). The number of intracellular bacteria in treated HepG2 cells was determined (C). Bacterial intracellular location was determined by immunofluorescent staining with a *Salmonella*-specific antibody (D).

(Fig. 2B). After intracellular bacteria were released, the number of colony-forming units (CFU) of bacteria was positively correlated to the intracellular bioluminescence (Fig. 2C). In addition, immunofluorescent staining showed that bacteria were localized mainly in the cytoplasm (Fig. 2D). These results showed that attenuated *S. typhimurium* SL7207-*lux* can rapidly enter HepG2 cells and that invasion correlates with the size of the inoculation.

3.2. *SL7207-lux inhibited the proliferation of human hepatoma HepG2 cells*

To explore the anti-proliferative effect of SL7207-*lux* on human hepatoma HepG2 cells, HepG2 cells were exposed to SL7207-*lux* at different multiplicities of infection (MOI) for 24, 48 and 96 h, respectively. SRB assay results showed that SL7207 treatment inhibited cell proliferation in HepG2 cells. The inhibitory rate of different MOI (1:25, 1:225, 1:675, 1:2025) was from 8 to 40% with 48-h treatment, and the highest inhibitory rate was increased up to 60% after 96-h prolonged treatment (Fig. 3).

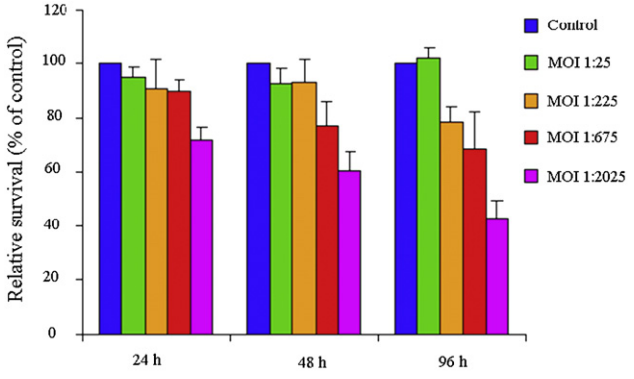

Figure 3 *In vitro* effects of SL7207-*lux* on human hepatoma HepG2 cells. Cells were exposed to SL7207 at different MOI for 24, 48 and 96 h, respectively, and cell survival was measured by SRB assay.

3.3. *SL7207-lux induced HepG2 cell apoptosis*

To determine whether *S. typhimurium* SL7207 induced HepG2 cell apoptosis, a TUNEL assay was done after 48-h treatment of SL7207-*lux*. The results showed a significant increase in the percentage of TUNEL positive cells after SL7207-*lux* treatment (Fig. 4A). This result was confirmed by a caspase-3 activity assay, which showed SL7207 markedly induced activation of caspase-3 (Fig. 4B). Taken together, we conclude that SL7207-*lux* treatment reduced cell viability of HepG2 cells through apoptosis.

3.4. *Optical imaging in living animals*

In order to observe *in vivo* real-time invasion of attenuated *S. typhimurium* SL7207 into tumor, an optical imaging system was used to track SL7207-*lux* in a hepatoma HepG2 xenograft model. At different times after SL7207-*lux* inoculation, bioluminescence of the nude mice could be detected. The results showed that the bacteria concentrated in the mouse liver area at 20 min,

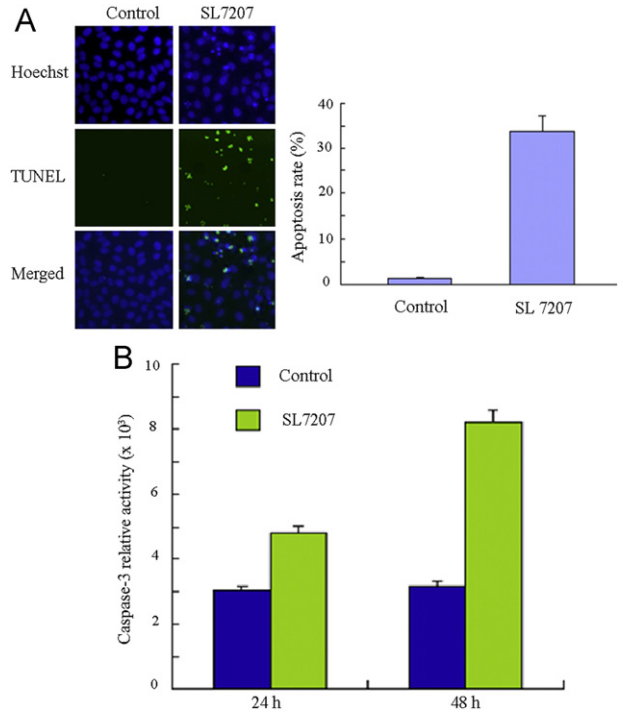

Figure 4 SL7207-*lux* induced HepG2 cell apoptosis. Cells were treated with *S. typhimurium* strain SL7207-*lux* (MOI 1:2025) for the indicated times and the TUNEL assay was measured to calculate the rate of apoptosis (apoptotic cells/total cells) (A) and caspase-3 activity assay (B).

and then gradually relocated to the tumor site (day 1–3). Five days later, the number of bacteria in the tumor site was about 400- to 1000-fold higher than in liver and other areas (Fig. 5). This result was concordant with the previous studies.

3.5. SL7207-lux inhibited the growth of hepatoma HepG2 xenografts in nude mice

In order to evaluate the potential effect of SL7207-*lux* on tumor growth *in vivo*, hepatoma HepG2 xenograft tumors (about 100 mm^3) were treated intravenously with 5×10^6 SL7207-*lux* two times a week for 5 weeks. The results showed that SL7207-*lux* significantly reduced tumor volume by 36% TGI (tumor growth inhibition) and tumor weight by 42% TGI (Fig. 6A, C and Table 1). Compared with the control group, the body weight of treatment group was not significantly reduced after 34 days of treatment (Fig. 6B and Table 1).

Bacteria in the liver and tumor tissue were released and quantified as *in vitro*, and showed that the number of colony-forming units (CFU) per gram of bacteria within tumor tissue was 1000-fold higher than that of liver or spleen (Fig. 6D). As shown in Fig. 6E, the necrosis area of tumor tissue in the control group was less than in SL7207-*lux*-treated group.

4. Discussion

Hepatocellular carcinoma yields highly malignant tumors leaving the patients with an average survival time of less than one year. Most hepatic carcinoma patients are diagnosed at an

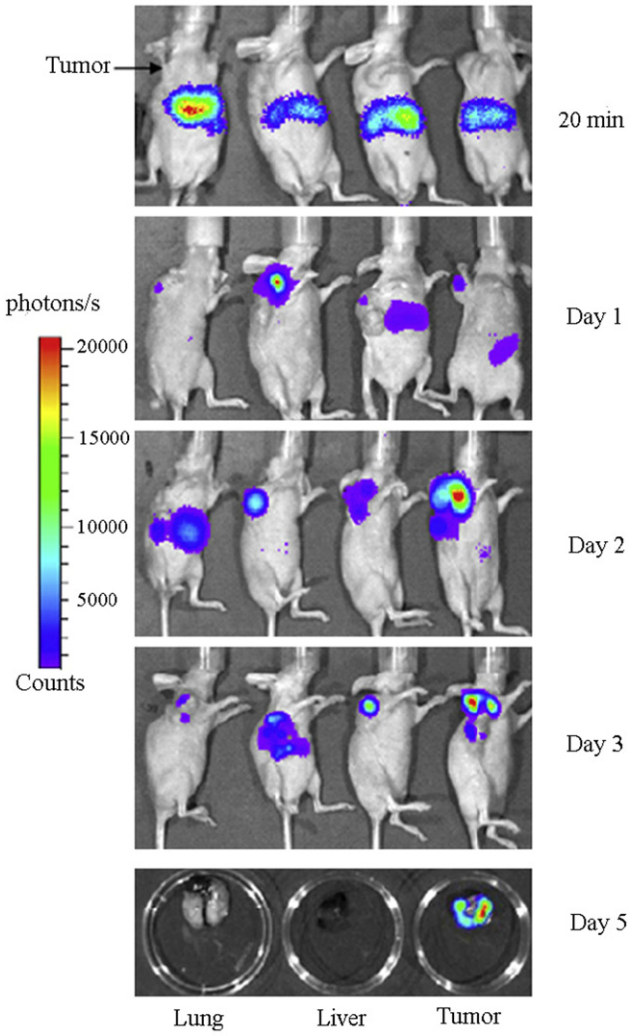

Figure 5 SL7207-*lux* proliferation and aggregation in human hepatoma HepG2 xenografts. After SL7207-*lux* inoculation for the indicated times, the bioluminescence of the nude mice was monitored by optical imaging system to track the bacteria. Five days after inoculation an anatomic observation was done to check the number of bacteria in the tumor, lung and liver (bottom).

advanced stage with cirrhosis and are not eligible for surgery. For those patients who cannot undergo surgery and liver transplantation, development of new therapeutic strategies which are efficient and specific but less toxic is of high priority[15].

In recent years different types of bacteria targeting cancer have been explored and several have been tested in pre-clinical experiments and/or clinical trials. Among those, *Salmonella enterica serovar typhimurium* (*S. typhimurium*) is a promising potential selection, and some genetically modified strains have been produced to yield better therapeutic effects and fewer side effects. *Salmonella* strains are highly immunologic and may induce septic shock mainly caused by the presence of lipid A[16]. The *Salmonella* strain VNP20009, which carries genomic deletions of *msbB* (gene product involved in synthesizing lipopolysaccharide) and *purI* to reduce septic shock potential while retaining tumor-targeting *in vivo* has been widely used in anticancer therapeutic studies. However, these studies have not been able to demonstrate a

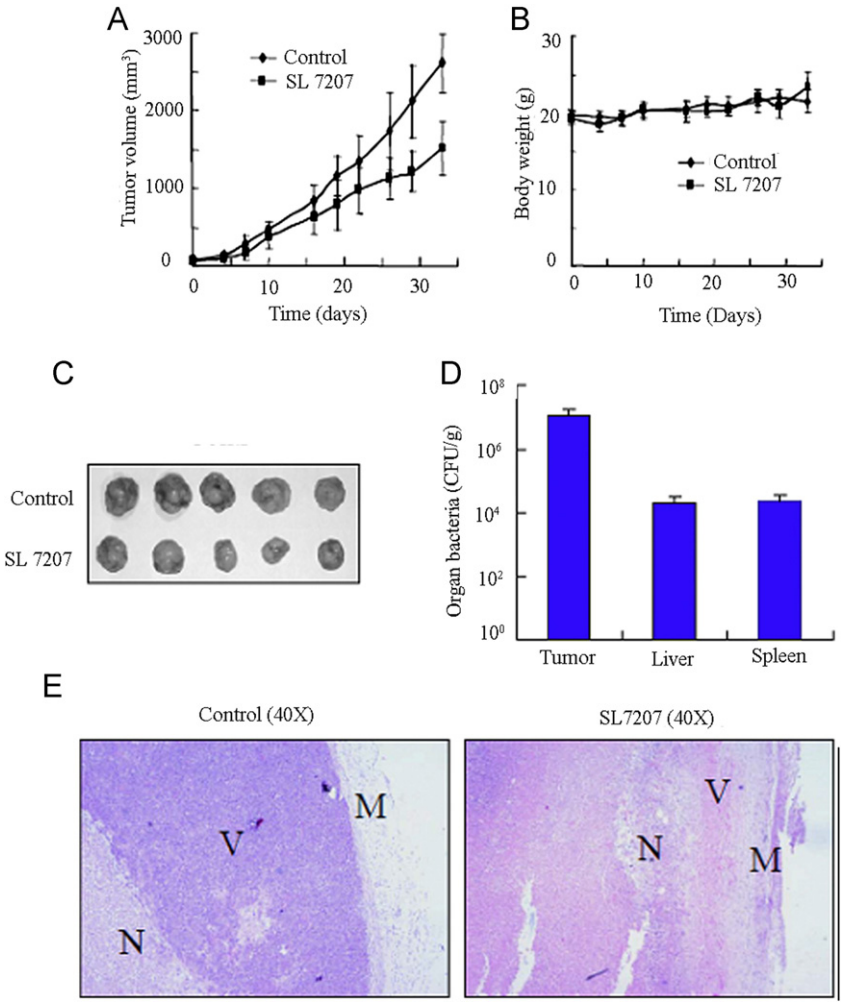

Figure 6 SL7207-*lux* restrained the growth of human hepatoma HepG2 xenografts. Mice with xenograft hepatoma HepG2 cell tumors were treated intravenously with SL7207-*lux* twice a week for 5 weeks. Tumor volume (A) and mouse body weight (B) was measured twice a week. At day 34, mice were killed and tumors were isolated (C). Liver, spleen and half of each tumor were homogenized to release and quantify the bacteria as *in vitro* (D). The remaining half of each tumor was used for HE staining (E). N: necrosis region; V: vital tumor rim; M: margin.

Table 1 Anti-tumor effects of SL7207-*lux* on tumor volume of human hepatoma HepG2 tumor-bearing nude mice.

Group	Mean weight (g)		Mean tumor volume (mm^3)
	Mouse	Tumor	
Control	21.47±1.54	2.38±0.38	2614.99±380.23
SL7207-*lux*	23.27±1.93	1.52±0.21*	1512.41±351.15*
TGI	–	36%	42%

*$P<0.05$ *vs.* control; TGI: tumor growth inhibition, $n=6$.

therapeutic effect. This might be due to the over-attenuation of VNP20009, thereby hampering entry of the bacteria into the tumors. The human immune system reacts more sensitively than that of the mouse to VNP20009 and rapidly clears it from the blood. SL7207, another genetically modified *S. typhimurium* strain which is less attenuated than VNP20009, has retained more tumor-toxic characteristics and may have better therapeutic effects. In this study, we investigated the antitumor activity of SL7207-*lux* both *in vitro* and *in vivo*, and found that it effectively induced cancer cell apoptosis. We also monitored the ability of the bacteria to invade hepatoma cells *in vitro* and *in vivo*.

Bacteria normally would be cleared quickly by the immune system in immune-competent mice. But necrotic areas of tumors have a high concentration of nutrients and lack of immunologic surveillance, providing a wonderful place for auxotrophic *Salmonella* growth and survival. Bacterial growth in tumor necrotic areas damaged surrounding tumor tissue and inhibited tumor growth. In this study, though immune-deficient nude mice were used, *Salmonella* could still be enriched in tumor sites and inhibit tumor growth, which also has been shown by at least one other *in vivo* study[8], indicating that bacteria in the mice had been cleared by mechanisms other than the immune system and might be worthy of further study.

Bacterial invasion and survival within cells is a very complicated process. Some studies have shown that low concentrations of bacteria are degraded by tumor cells, but

high concentrations of bacteria permit entry into cells, leading to cell lysis[5,17]. In this study, cell proliferation studies showed that low concentrations of bacteria did not significantly affect cell growth. *In vivo* studies showed that the animal body weight also was not affected by bacteria, indicating that SL7207-*lux* had low toxicity when the dosage was low. When the concentration of bacteria was high, cell apoptosis was induced and resulted in the inhibition of cell proliferation. Bacteria enriched in the tumor area (about 1000-fold higher than in liver and spleen tissues) led to about 40% tumor growth inhibition *in vivo*, which was consistent with the high concentrations of bacteria *in vitro*.

In this study, a bacterial luminescent gene cluster was transfected into attenuated *S. typhimurium* SL7207-*lux* cells and *in vitro* and *in vivo* invasion of bacteria into hepatoma cells was monitored. SL7207-*lux* showed significant effects on human hepatoma HepG2 cells *in vitro* and *in vivo* without toxicity, indicating that *Salmonella* might be developed into a tool for cancer therapy. With this in mind, SL7207 could be developed into RNAi-based anticancer vector.

Acknowledgements

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References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011;**61**:69–90.
2. Chan SL, Yeo W. Targeted therapy of hepatocellular carcinoma: present and future. *J Gastroenterol Hepatol* 2012;**27**:862–72.
3. Pawelek JM, Low KB, Bermudes D. Tumor-targeted *Salmonella* as a novel anticancer vector. *Cancer Res* 1997;**57**:4537–44.
4. Yam C, Zhao M, Hayashi K, Ma H, Kishimoto H, McElroy M, et al. Monotherapy with a tumor-targeting mutant of *S. typhimurium* inhibits liver metastasis in a mouse model of pancreatic cancer. *J Surg Res* 2010;**164**:248–55.
5. Leschner S, Weiss S. *Salmonella*-allies in the fight against cancer. *J Mol Med (Berl)* 2010;**88**:763–73.
6. Hoiseth SK, Stocker BA. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* 1981;**291**:238–9.
7. Yu B, Yang M, Shi L, Yao YD, Jiang QQ, Li XF, et al. Explicit hypoxia targeting with tumor suppression by creating an ''obligate'' anaerobic *Salmonella typhimurium* strain. *Sci Rep* 2012;**2**:436.
8. Guo H, Zhang J, Inal C. Targeting tumor gene by shRNA-expressing *Salmonella*-mediated RNAi. *Gene Ther* 2011;**18**:95–105.
9. Samonis G, Maraki S, Kouroussis C, Mavroudis D, Georgoulias V. *Salmonella enterica pneumonia* in a patient with lung cancer. *J Clin Microbiol* 2003;**41**:5820–2.
10. Ahmad S, Casey G, Cronin M, Rajendran S, Sweeney P, Tanqney M, et al. Induction of effective antitumor response after mucosal bacterial vector mediated DNA vaccination with endogenous prostate cancer specific antigen. *J Urol* 2011;**186**:687–93.
11. Fest S, Huebener N, Bleeke M, Durmus T, Stermann A, Woehler A, et al. Survivin minigene DNA vaccination is effective against neuroblastoma. *Int J Cancer* 2009;**125**:104–14.
12. Paglia P, Medina E, Arioli I, Guzman CA, Colombo MP. Gene transfer in dendritic cells, induced by oral DNA vaccination with *Salmonella typhimurium*, results in protective immunity against a murine fibrosarcoma. *Blood* 1998;**92**:3172–6.
13. Zhang SH, Zhang H, He HW, Li Y, Li XY, Zhang LF, et al. *In vivo* real-time imaging of gemcitabine-leaded growth inhibition in the orthotopic transplantation model of human pancreatic tumor. *Acta Pharm Sin B* 2011;**1**:220–5.
14. Sun HX, He HW, Zhang SH, Liu TG, Ren KH, He QY, et al. Suppression of N-Ras by shRNA-expressing plasmid increases sensitivity of HepG2 cells to vincristine-induced growth inhibition. *Cancer Gene Ther* 2009;**16**:693–702.
15. Llovet JM, Beaugrand M. Hepatocellular carcinoma: present status and future prospects. *J Hepatol* 2003;**38**:S136–49.
16. Low KB, Ittensohn M, Le T, Platt J, Sodi S, Amoss M, et al. Lipid A mutant *Salmonella* with suppressed virulence and TNFalpha induction retain tumor-targeting *in vivo*. *Nat Biotechnol* 1999;**17**:37–41.
17. dos Santos SA, de Andrade DR Jr., de Andrade DR. TNF-alpha production and apoptosis in hepatocytes after *Listeria* monocytogenes and *Salmonella typhimurium* invasion. *Rev Inst Med Trop Sao Paulo* 2011;**53**:107–12.