Original Article

In vivo Bioluminescence-Based Monitoring of Liver Metastases from Colorectal Cancer: An Experimental Model

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Abstract

Background: In this study we aimed to develop a new *in vivo* bioluminescence-based tool to monitor and to quantify colon cancer (CC) liver metastasis development. **Methods:** HCT 116 cells were transducted with pLenti6/V5-DEST-fLuc for constitutive expression of firefly luciferase. Infection was monitored analyzing endogenous bioluminescence using the IVIS Lumina II *In vivo* Imaging System and a positive clone constitutively expressing luciferase (HCT 116-fLuc) was isolated. HCT 116-fLuc cells were left untreated or treated with 1 µM GDC-0449, a Hedgehog pharmacological inhibitor. Moreover, 1 x 106 HCT 116-fLuc cells were implanted via intra-splenic injection in nude mice. Bioluminescence was analyzed in these mice every 7 days for 5 weeks. After that, mice were sacrificed and bioluminescence was analyzed on explanted livers. **Results:** We found that *in vitro* bioluminescence signal was significantly reduced when HCT 116-fLuc cells were treated with GDC-0449. Regarding *in vivo* data, bioluminescence sources consistent with hepatic anatomical localization were detected after 21 days from HCT 116-fLuc intrasplenic injection and progressively increased until the sacrifice. The presence of liver metastasis was further confirmed by *ex-vivo* bioluminescence analysis of explanted livers. **Conclusions:** Our *in vitro* results suggest that inhibition of Hedgehog pathway may hamper CC cell proliferation and impel for further studies. Regarding *in vivo* data, we set-up a strategy for liver metastasis visualization, that may allow follow-up and quantification of the entire metastatic process. This cost-effective technique would reduce experimental variability, as well as the number of sacrificed animals.

Keywords: Cell invasiveness, cell plasticity, colon cancer, colorectal cancer, hedgehog pathway, liver metastasis

Introduction

Colon cancer (CC) represents the second leading cause of death in the US and is up-to-now the fourth most frequently diagnosed malignant disease. It has been reported that in Italy, colon and rectum were the most frequent cancer sites in 2012, accounting for over 54,000 new diagnoses.[1,2] Several molecular pathways control cellular differentiation and proliferation; their activation or deregulation plays a role in the development and progression of both familiar and sporadic cases of CC.[3,4] Due to the poor results for metastatic CC with current chemotherapy protocols, the analysis of novel pathways playing a role in the pathogenesis of CC is an active field of research. It has been reported that 50%–60% of patients affected by CC develop metastases, and, in particular, 20%–34% of them present with synchronous metastases.[5,6]

It has been demonstrated that the presence of active hedgehog (Hh)-GLI activity in the epithelial tumor cells of colorectal cancer (CRC) is essential for tumor growth, recurrence, and metastatic growth and regulates the behavior of human CC stem cells *in vivo*.[7] Therefore, we can postulate that modulating Hh

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pathway may interfere with the metastatic spread. As reported by Sicklick *et al*., hepatic stellate cells (HSCs) show Hh activity in their activated phenotype.[8] Our previously reported data have demonstrated that hepatocellular carcinoma regulates HSCs' viability via paracrine signaling by modulating Hh pathway.[9] Apparently, the regulation of Hh pathway influences both tumor-stroma crosstalk and tumor growth. Taken together, these data suggested that the use of a Hh inhibitor may interfere with the metastatic spread and in particular with the liver localization of metastases from CC. Moreover, we demonstrated in an *in vitro* experimental system that Hh pathway fosters cell invasion integrating cell proliferation, cell plasticity, and glucose/amino acids metabolism (Magistri *et al*. submitted).[10] To elucidate the mechanisms leading to liver metastasis and to provide preclinical tools of investigation for innovative therapies, suitable animal models of CRC[11] with liver metastasis[12] have been developed. Here, we describe a murine model of CC metastatic to the liver[13] and evaluate the progression of the disease through an *in vivo* bioluminescence-based monitoring of the metastasis. In particular, we have obtained a xenograft mouse model of CRC metastasis based on the intrasplenic injection of the human CC HCT 116 cells.[14] The feasibility of this model, along with the histologic evidence of liver metastasis, will allow further applications of the protocol, to test the efficacy of our therapeutic regimen, reducing the number of sacrificed animals, with all its ethical and economic implications.

Materials and Methods

Cell culture conditions

HCT 116 human CC cell line was grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO® Life Technology, Monza, Italy) and antibiotics. Where reported, cells were treated with Hh inhibitor GDC-0449 (Genentech, Inc., San Francisco, CA, USA) 1 μM for 24 or 48 h.

Lentiviral vectors

We used the third-generation self-inactivating lentiviral vectors derived by pLenti6/V5-DEST (Invitrogen, Carlsbad, CA, USA) for the expression of luciferase (firefly luciferase, fLuc) (LV-fLuc). To obtain viral particles, 8.0×10^6 293-T cells were seeded into a 15-cm tissue culture plate. The day after, cells were cotransfected using the calcium phosphate coprecipitation method with the pLenti6/V5-DEST-fLuc plasmid (25 μg), in combination with plasmids that incorporate transfunctions needed for virus packaging (pMDL, 12.5 μg; pREV, 6.2 μg; pVSVG, 9 μg). Calcium phosphate-precipitated DNAs were removed after 16 h by replacing the culture medium. After 48 h, cell supernatant containing the viral particles was collected.[15]

Viral-mediated gene transfer in HCT 116 cells

HCT 116 cells were seeded at density of 4.0×10^4 cells/cm^2 and cultured in DMEM + 10% FBS in standard conditions. After 1 day, when appearing to be confluent at 60%, they were transduced with LV-fLuc (10 transforming units/cell) in the presence of 6 μg/ml polybrene. After 2 h incubation at 37°C, fresh medium was added. The following day cells were trypsinized and subcultured at 1:3. Blasticidin (5 μg/ml) was added to the culture medium 48 h after transduction to select infected cells. Transduced cells were monitored by bioluminescence analysis (see below), and a clone of HCT 116 cells stably expressing luciferase (HCT 116-fLuc) was isolated.

Intrasplenic injection

Procedures involving mice were performed according to the Guidelines of the National Institutes of Health and Current National Legislation (European Directive 2010/63 125 UE, Italian D. Lgs 26/2014), in conformity to the procedures of the Institutional Animal Care and Use Committee. Animals used in the study were 8-week-old nu/nu male mice (Envigo, Italy) housed in individual ventilated cages in a facility with constant temperature and a 12-h light cycle. Infected HCT 116 cells (HCT 116-fLuc) were implanted via intrasplenic injection into a group of six nude mice (1×10^6 cells/mouse in 100 μl of physiological solution). Mice were anesthetized with Xilor-100/Zoletil (2 mg/kg) by intramuscular administration. A 1 cm laparotomy was then performed in the left subcostal region of the abdomen, and the spleen was gently exposed and the cells injected with a 27G needle. The spleen was then put back into the abdominal cavity and the abdominal wall sutured with stitches.

In vitro, *ex vivo,* and *in vivo* bioluminescence analysis

In vitro (cells in culture), *ex vivo* (harvested organs and tissues), and *in vivo* (living mouse) bioluminescence analysis was performed using the IVIS Lumina II *in vivo* imaging system (PerkinElmer, Waltham, MA, USA) as previously described.[16] For *in vitro* analysis, HCT 116 cells were cultured on plastic dishes (BD, Franklin Lakes, NJ, USA), then incubated with media in the presence of D-luciferin (PerkinElmer) (150 μg/ml) for 5 min, and then analyzed. The procedure was similar for bioptic samples: Tissues were washed in phosphate-buffered saline (PBS), incubated for 5 min in the presence of D-luciferin (150 μg/ml) dissolved in PBS, and then analyzed. For *in vivo* analysis, animals were anesthetized by intraperitoneal injection of avertin (200 mg/kg). Luciferin dissolved in PBS (150 mg/kg) was also administered intraperitoneally. After 10 min, the animal was put into the detection system, and the signal was acquired in a time range of 1–5 min, depending on signal intensity. Living Image Software (PerkinElmer, Waltham, MA, USA) was used to analyze the signals in manually selected regions of interest. Data were expressed as photons per second per square centimeter per steradian (p/s/cm^2/sr).

Results

Generation of HCT 116-fLuc cells and visualization of luciferase activity

We used a third-generation lentiviral vector expressing luciferase

to permanently mark live HCT 116 cells. HCT 116 cells were genetically modified to constitutively harbor luciferase activity. After an initial selection of luciferase-positive clones with blasticidin, luciferase expression was maintained for several passages (data not shown). Bioluminescence analysis on subconfluent dishes of HCT 116-infected cells demonstrated a generation of an endogenous bioluminescent signal, achieving, in detail, an emission of 6.9×10^6 photons/s/cm²/sr [Figure 1].

In vitro analysis of the effect of GDC-0449 in controlling HCT 116-fLuc cell proliferation/viability

Emission of photons by luciferase-expressing cells is based on the oxidation of the substrate D-luciferin, a reaction that requires oxygen, Mg^{2+}, and ATP. Therefore, since generation of bioluminescence signal by luciferase is linked to cellular ATP consumption, we assumed that cellular metabolic status positively correlated with the bioluminescence imaging (BLI) signal emission by HCT 116-fLuc cells.

To evaluate the possible effect of pharmacological Hh inhibition in cellular viability, HCT 116-fLuc cells were cultured either in cell culture medium alone or supplemented with vehicle (dimethyl sulfoxide) or with GDC-0449 (1 μM). The bioluminescent signal was evaluated before exposure to GDC-0449 (0 h) and after 48 h of treatment and compared with vehicle/nontreated cells. We observed a statistically significant reduction in the BLI signal emitted by cells in the presence of GDC-0449, in comparison to controls [Figure 2]. These data suggest that the pharmacological inhibition of the Hh pathway impairs HCT 116-fLuc cell proliferation/viability.

Dynamic *in vivo* analysis of HCT 116-fLuc cell liver metastasization process in mice

HCT 116-fLuc cells were also used in an *in vivo* setting to follow the process of liver metastasization and tumor mass formation after their injection into the spleen of nude mice. *In vivo* BLI after intrasplenic injection[17] of 1×10^6 HCT 116-fLuc demonstrated cell growth and the development of a tumor mass at the site of delivery, starting at 3 weeks and becoming more evident at 4 weeks after the injection [Figure 3]. Moreover, we detected bioluminescence sources consistent with hepatic anatomical localization [Figure 4].

Macroscopic analysis of the liver after necropsy of mice performed 5 weeks after HCT 116-fLuc administration revealed multiple areas with small tumor growth [Figure 5, left panel]. The presence of liver metastasis was confirmed by *ex vivo* bioluminescence analysis of the livers [Figure 5, right panel]. Moreover, in accordance with macroscopic observation and BLI assessment, also histological analyses confirmed the presence of liver metastasis (data not shown). Overall, we set up a bioluminescence-based toll providing quantitative information *in vitro* on cell viability and valuable information for the follow-up of the progression of metastatic process *in vivo*.

In other terms, in this model BLI can be used to easily predict the presence of liver metastasis without the sacrifice of the mouse. This allows for possible use of this strategy for evaluating pharmacological treatments aiming to the reduction of CC metastatic spreading to the liver.

Discussion

It has been estimated that patients affected by CC develop metastatic disease in 50%–60% of cases, either synchronous or metachronous. Among them, 80%–90% of liver diseases are not amenable to surgical resection.[18] This is extremely relevant for the natural history of the disease, since surgical resection of colorectal liver metastases is related to improved survival, while a longer interval from diagnosis to resection is associated with worse overall survival.[19] During the last two decades, staged

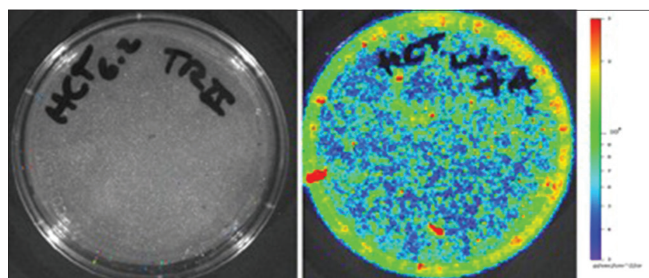

Figure 1: HCT 116 cells are prone to lentiviral-mediated firefly luciferase gene transfer. *In vitro* bioluminescence analysis of HCT 116 cells after transduction with lentiviral vectors that express luciferase. HCT 116-fLuc cells (1.8×10^6) were counted and plated in a 100-mm tissue culture plate. Bioluminescence imaging was performed after 24 h. The image shows a representative image of a plate with nontransduced cells (left) and of a plate with cells transduced as above (right). The color bar image indicates the relative bioluminescent signal intensities from the lowest (blue) to the highest (red). Values are expressed in photons per second per square centimeter per steradian (photons/s/cm²/sr)

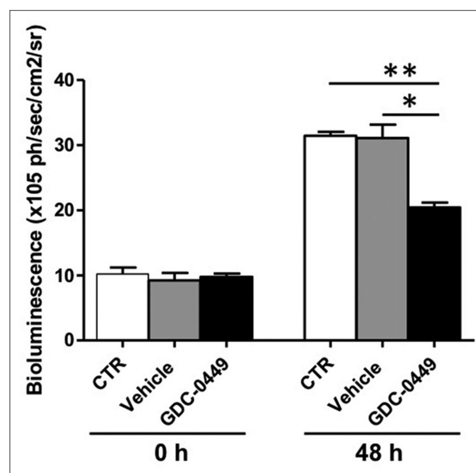

Figure 2: HCT 116 cells proliferation/viability is affected by treatment with a pharmacological inhibitor of Hedgehog quantification of bioluminescence emitted by HCT 116-fLuc, assessed after 0 and 48 h of culture in the presence of GDC-0449 (1 μM). Data are expressed as means ± standard error from three independent experiments. Asterisk (*) indicates a significant difference versus both the control and the "Vehicle" group, assessed by a two-tailed Student's *t*-test for paired data; statistical significance level was set at $P < 0.05$

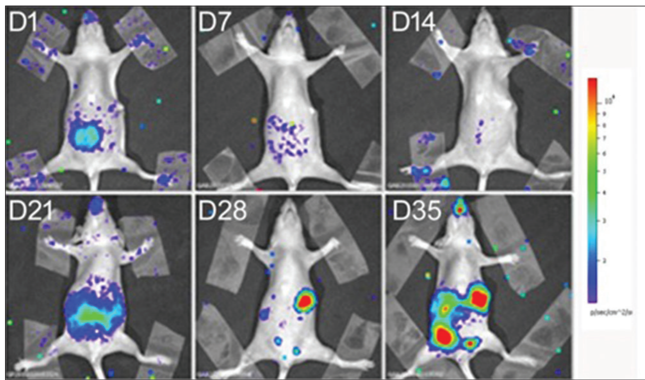

Figure 3: Progression of tumor burden by bioluminescence imaging bioluminescence imaging longitudinal analysis of a representative mouse at different time points (1, 7, 14, 21, 28, and 35 days, respectively) after intrasplenic injection of 1 × 10^6 HCT 116-fLuc cells

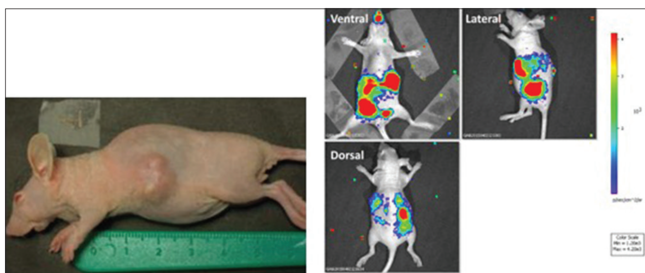

Figure 4: Assessment of tumor spread by *in vivo* bioluminescence imaging. Photographic image (left) and bioluminescence imaging analysis (right) of a mouse 5 weeks after intrasplenic injection of 1 × 10^6 HCT 116-fLuc cells

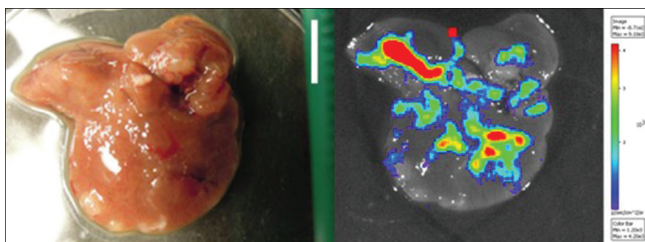

Figure 5: Assessment of hepatic metastatization by *ex vivo* bioluminescence imaging. Photographic image and bioluminescence imaging analysis of the liver explanted from a mouse 5 weeks after intrasplenic injection of 1 × 10^6 HCT 116-fLuc cells. Macroscopic liver analysis. Left panel: Image of the liver acquired with a digital camera. Right panel: Bioluminescence image of the same sample superimposed to photographic image acquired by the IVIS Lumina II Imaging System. Scale bar: 0.5 cm

surgical approaches such as two-stage hepatectomy (TSH) and associating liver partition and portal vein ligation for staged hepatectomy have been developed to manage patients with initially unresectable liver disease, i.e., bilobar metastases or insufficient future liver remnant.[20,21] About TSH, it has been reported a rate of 23% of median failure of completing the two-stage approach (range 0%–36%), mostly due to disease progression.[22] Therefore, some authors proposed that response to chemotherapy may be considered as a surrogate marker of disease severity. Conversely, Kishi *et al*. in their multicenter study demonstrated that prolonged preoperative FOLFOX therapy increases the risks of hepatotoxicity and postoperative hepatic insufficiency.[23] As a matter of fact, novel oncologic approaches are needed to boost the current surgical innovations.

Bioluminescent *in vivo* molecular imaging techniques make the most of highly sensitive tools equipped with charge coupled device (CCD) cameras, which when maintained at low temperatures can detect photons emitted from an appropriate light source.[24] Tumor cells act as light sources when they express a bioluminescent marker so that they can be tracked with *in vivo* imaging analysis.

These techniques may allow to perform longitudinal, dynamic observation at specific time intervals. It may allow to study animals *in vivo* after tumor injection, with quantitative and qualitative analysis of tumor growth and, consequently, of treatment efficacy. In this study, we analyze cells' properties such as migration, invasion, and proliferation using HCT 116 CRC stable cell line infected with LV-fLuc to constitutively express luciferase. To generate experimental liver metastasis *in vivo*, cells were administered by intrasplenic injection.[25]

Luciferase-expressing cells emit light by oxidation of the substrate luciferin, which can be conveniently administered to the mouse by intraperitoneal injection. Therefore, by noninvasive *in vivo* BLI, using a high-sensitive CCD camera, it is possible to detect and precisely quantify the photons emitted from cells expressing a luciferase enzyme. The intensity of the BLI signal correlates with the number of the luciferase-expressing cells and consequently with tumor mass burden.[26] Moreover, bioluminescence images acquired with the CCD camera can be superimposed on photographic images of the mouse with the purpose of anatomically identify the region of the emission. Furthermore, the bioluminescence signal to background ratio is low, thus permitting sensitive and quantitative analysis.[27] In addition, BLI is a noninvasive methodology making possible the repeated (longitudinal) assessment of tumor progression in a given animal, reducing the number of animals needed, thus lowering the costs and the ethical concerns associated with the use of animal in experimental procedures. However, it should be noted that the intensity of BLI is also dependent on the source of light localization within the body, with an approximate 10-fold decrease of intensity for each centimeter of tissue depth. Therefore, the detection of BLI signal emission generated by deeper liver metastasis is reduced with respect to signals arising from other more superficial regions.

Conclusions

Overall, we evaluated whether intrasplenic administration of HCT 116-fLuc cells can be instrumental in the analysis of CC progression and for mapping tumor cell dissemination to assess possible therapeutic efficacy of pharmacological modulation of Hh activity. These data are preliminary for the evaluation

of the role of a specific pharmacologic Hh inhibitor in the natural history of CC *in vivo*. Our unpublished data, in fact, suggest that modulating the Hh pathway may interfere with the metastatic process of the disease, altering cell proliferation, cell plasticity, and glucose/amino acid metabolism.

Acknowledgments

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Conflicts of interest

There are no conflicts of interest.

References

1. Ciatto S. Current cancer profiles of the Italian regions: Should cancer incidence be monitored at a national level? Tumori 2007;93:529-31.
2. Rossi S, Crocetti E, Capocaccia R, Gatta G; AIRTUM Working Group. Estimates of cancer burden in Italy. Tumori 2013;99:416-24.
3. Al-Sohaily S, Biankin A, Leong R, Kohonen-Corish M, Warusavitarne J. Molecular pathways in colorectal cancer. J Gastroenterol Hepatol 2012;27:1423-31.
4. Bertrand FE, Angus CW, Partis WJ, Sigounas G. Developmental pathways in colon cancer: Crosstalk between WNT, BMP, Hedgehog and Notch. Cell Cycle 2012;11:4344-51.
5. Van Cutsem E, Nordlinger B, Adam R, Köhne CH, Pozzo C, Poston G, *et al.* Towards a pan-European consensus on the treatment of patients with colorectal liver metastases. Eur J Cancer 2006;42:2212-21.
6. Muratore A, Zorzi D, Bouzari H, Amisano M, Massucco P, Sperti E, *et al.* Asymptomatic colorectal cancer with un-resectable liver metastases: Immediate colorectal resection or up-front systemic chemotherapy? Ann Surg Oncol 2007;14:766-70.
7. Gupta S, Takebe N, Lorusso P. Targeting the hedgehog pathway in cancer. Ther Adv Med Oncol 2010;2:237-50.
8. Sicklick JK, Li YX, Choi SS, Qi Y, Chen W, Bustamante M, *et al.* Role for hedgehog signaling in hepatic stellate cell activation and viability. Lab Invest 2005;85:1368-80.
9. Magistri P, Leonard SY, Tang CM, Chan JC, Lee TE, Sicklick JK. The glypican 3 hepatocellular carcinoma marker regulates human hepatic stellate cells via hedgehog signaling. J Surg Res 2014;187:377-85.
10. Magistri P, Battistelli C, Strippoli R, Petrucciani N, Pellinen T, Rossi L, *et al*. SMO Inhibition Modulates Cellular Plasticity and Invasiveness in Colorectal Cancer. Front Pharmacol 2018;8:956.
11. Evans JP, Sutton PA, Winiarski BK, Fenwick SW, Malik HZ, Vimalachandran D, *et al.* From mice to men: Murine models of colorectal cancer for use in translational research. Crit Rev Oncol Hematol 2016;98:94-105.
12. Oh BY, Hong HK, Lee WY, Cho YB. Animal models of colorectal cancer with liver metastasis. Cancer Lett 2017;387:114-20.
13. Hackl C, Man S, Francia G, Milsom C, Xu P, Kerbel RS. Metronomic oral topotecan prolongs survival and reduces liver metastasis in improved preclinical orthotopic and adjuvant therapy colon cancer models. Gut 2013;62:259-71.
14. Rajput A, Dominguez San Martin I, Rose R, Beko A, Levea C, Sharratt E, *et al.* Characterization of HCT116 human colon cancer cells in an orthotopic model. J Surg Res 2008;147:276-81.
15. Tiscornia G, Singer O, Verma IM. Production and purification of lentiviral vectors. Nat Protoc 2006;1:241-5.
16. Di Rocco G, Gentile A, Antonini A, Truffa S, Piaggio G, Capogrossi MC, *et al.* Analysis of biodistribution and engraftment into the liver of genetically modified mesenchymal stromal cells derived from adipose tissue. Cell Transplant 2012;21:1997-2008.
17. Heijstek MW, Kranenburg O, Borel Rinkes IH. Mouse models of colorectal cancer and liver metastases. Dig Surg 2005;22:16-25.
18. Kemeny N. Management of liver metastases from colorectal cancer. Oncology (Williston Park) 2006;20:1161-76, 1179.
19. Leal JN, Bressan AK, Vachharajani N, Gonen M, Kingham TP, D'Angelica MI, *et al.* Time-to-surgery and survival outcomes in resectable colorectal liver metastases: A multi-institutional evaluation. J Am Coll Surg 2016;222:766-79.
20. Adam R, Laurent A, Azoulay D, Castaing D, Bismuth H. Two-stage hepatectomy: A planned strategy to treat irresectable liver tumors. Ann Surg 2000;232:777-85.
21. Schnitzbauer AA, Lang SA, Goessmann H, Nadalin S, Baumgart J, Farkas SA, *et al.* Right portal vein ligation combined with *in situ* splitting induces rapid left lateral liver lobe hypertrophy enabling 2-staged extended right hepatic resection in small-for-size settings. Ann Surg 2012;255:405-14.
22. Chua TC, Liauw W, Chu F, Morris DL. Summary outcomes of two-stage resection for advanced colorectal liver metastases. J Surg Oncol 2013;107:211-6.
23. Kishi Y, Zorzi D, Contreras CM, Maru DM, Kopetz S, Ribero D, *et al.* Extended preoperative chemotherapy does not improve pathologic response and increases postoperative liver insufficiency after hepatic resection for colorectal liver metastases. Ann Surg Oncol 2010;17:2870-6.
24. Xu T, Close D, Handagama W, Marr E, Sayler G, Ripp S. The expanding toolbox of *in vivo* bioluminescent imaging. Front Oncol 2016;6:150.
25. Lafreniere R, Rosenberg SA. A novel approach to the generation and identification of experimental hepatic metastases in a murine model. J Natl Cancer Inst 1986;76:309-22.
26. Klerk CP, Overmeer RM, Niers TM, Versteeg HH, Richel DJ, Buckle T, *et al.* Validity of bioluminescence measurements for noninvasive *in vivo* imaging of tumor load in small animals. Biotechniques 2007;43:7-13, 30.
27. Thalheimer A, Korb D, Bönicke L, Wiegering A, Mühling B, Schneider M, *et al.* Noninvasive visualization of tumor growth in a human colorectal liver metastases xenograft model using bioluminescence *in vivo* imaging. J Surg Res 2013;185:143-51.