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Original Article

In vivo bioluminescence-based monitoring of liver metastases from colorectal cancer: An experimental model

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ABSTRACT

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. 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, 1×10^6 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. 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. 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.

1. 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 site in 2012, accounting for over 54,000 new diagnoses [1,2]. Several molecular pathways control cellular differentiation and proliferation; their activation or deregulation play 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 epithelial tumor cells of colorectal cancer 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 pathway may interfere with the metastatic spread. As reported by Sicklick and colleagues, Hepatic Stellate Cells (HSCs) show Hh activity in their activated phenotype [8]. Our previously reported data have demonstrated that Hepatocellular Carcinoma (HCC) 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 an Hh inhibitor may interfere with the metastatic spread and in particular with

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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/aminoacids metabolism (Magistri et al. submitted). In order to elucidate the mechanisms leading to liver metastasis and to provide preclinical tools of investigation for innovative therapies, suitable animal models of colorectal cancer [10] with liver metastasis [11] have been developed. Here, we describe a murine model of CC metastatic to the liver [12], 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 colorectal cancer metastasis based on the intra-splenic injection of the human colon cancer HCT 116 cells [13]. 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.

2. Materials and methods

2.1. Cell culture conditions

HCT 116 human colon cancer cell line was grown in DMEM supplemented with 10% FBS (GIBCO® Life Technology, Monza, Italy) and antibiotics. Where reported, cells were treated with Hedgehog Inhibitor GDC-0449 (GDC-0449) (Genentech, Inc., San Francisco, CA, USA) 1 μM for 24 or 48 h.

2.2. Lentiviral vectors

We used third generation self-inactivating lentiviral vectors derived by pLenti6/V5-DEST (Invitrogen, Carlsbad, CA) for 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 co-transfected using the calcium phosphate co-precipitation method with the pLenti6/V5-DEST-fLuc plasmid (25 μg), in combination with plasmids that incorporate trans functions 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 [14].

2.3. Viral-mediated gene transfer in HCT 116 cells

HCT 116 were seeded at density of 4.0×10^4 cells/cm^2 and cultured in DMEM + 10% FBS in standard conditions. After one day, when appearing to be confluent at 60%, they were transduced with LV-fLuc (10 transforming units/cell) in 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.

2.4. Intra-splenic 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 procedures of the Institutional Animal Care and Use Committee. Animals used in the study were 8 weeks 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* intra-splenic injection into a group of 6 nude mice (1×10^6 cells/mouse in 100 μl of physiological solution). Mice were anesthetized with Xilor-100/Zoletil (2 mg/kg) by intra-muscular (IM) administration. A 1 cm laparotomy was then performed in the left subcostal region of the abdomen and the spleen was gently exposed and cells injected with a 27 G needle. The spleen was then put back into the abdominal cavity and the abdominal wall sutured with stitches.

2.5. 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 [15]. 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 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 intra-peritoneal injection of Avertin (200 mg/kg). Luciferin dissolved in PBS (150 mg/kg) was also administered intra-peritoneally. Ten minutes later 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) 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/sec/cm^2/sr).

3. Results

3.1. 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 in order 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 sub-confluent 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/sec/cm^2/sr (Fig. 1).

3.2. 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 (DMSO) 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/non-treated cells. We observed a statistically sig-

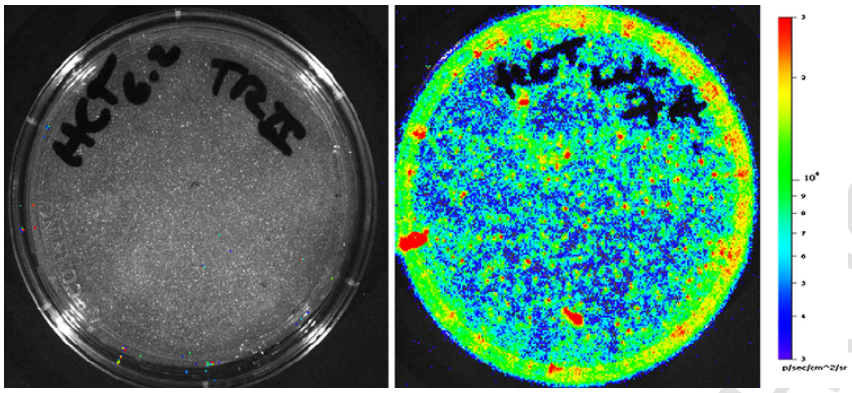

Fig. 1. HCT 116 cells are prone to lentiviral-mediated firefly luciferase gene transfer. *In vitro* bioluminescence analysis of HCT 116 cells after transduction with lentiviral vectors that express luciferase. HCT 116-fLuc cells (1.8×10^6) were counted and plated in a 100-mm tissue culture plate. Bioluminescence imaging was performed after 24 h. The image shows a representative image of a plate with non-transduced 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/cm^2/sr).

nificant reduction in the BLI signal emitted by cells in presence of GDC-0449, in comparison to controls (Fig. 2). These data suggest that the pharmacological inhibition of the Hh pathway impairs HCT 116-fLuc cell proliferation/viability.

3.3. Dynamic in vivo analysis of HCT 116-fLuc cell liver metastatization process in mice

HCT 116-fLuc cells were also used in an *in vivo* setting to follow the process of liver metastatization and tumor mass formation after their injection into the spleen of nude mice. *In vivo* bioluminescent imaging after intra-splenic injection [16] 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 (Fig. 3). Moreover, we detected bioluminescence sources consistent with hepatic anatomical localization (Fig. 4).

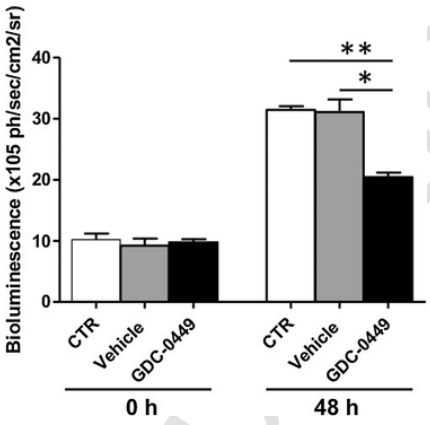

Fig. 2. HCT 116 cells proliferation/viability is affected by treatment with a pharmacological inhibitor of Hedgehog. Quantification of bioluminescence emitted by HCT 116-fLuc, assessed after 0 and 48 h of culture in the presence of GDC-0449 (1 μM). Data are expressed as means ± standard error from three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

Macroscopic analysis of the liver after necropsy of mice performed 5 weeks after HCT 116-fLuc administration revealed multiple areas with small tumor growth (Fig. 5, left panel). The presence of liver metastasis was confirmed by *ex-vivo* bioluminescence analysis of the livers (Fig. 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 setup 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 of bioluminescence imaging 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.

4. Discussion

It has been estimated that patients affected by CC develop a metastatic disease in 50% to 60% of cases, either synchronous or metachronous. Among them, 80%–90% of liver diseases are not amenable to surgical resection [17]. 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 [18]. During the last two decades, staged surgical approaches such as two-stage hepatectomy (TSH) and associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) have been developed to manage patients with initially unresectable liver disease, *i.e.* bilobar metastases or insufficient future liver remnant [19,20]. 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 [21]. 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 [22]. As a matter of fact, novel oncologic approaches are needed to boost current surgical innovations.

Bioluminescent *in vivo* molecular imaging techniques make the most of highly sensitive tools equipped with Charge Coupled Device

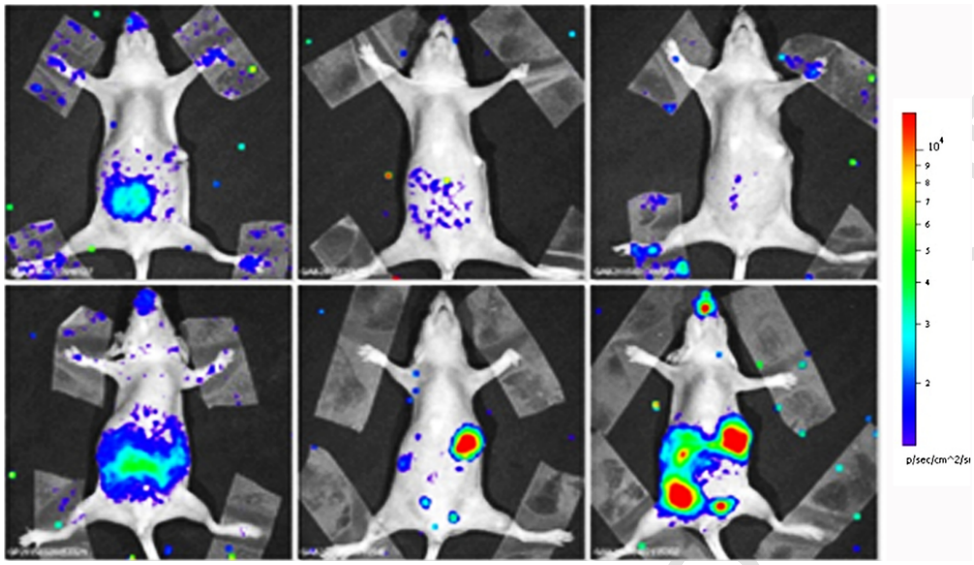

Fig. 3. Progression of tumor burden by bioluminescence imaging. BLI longitudinal analysis of a representative mouse at different time points (1, 7, 14, 21, 28, 35 days respectively) after intra-splenic injection of 1×10^6 HCT 116-fLuc cells.

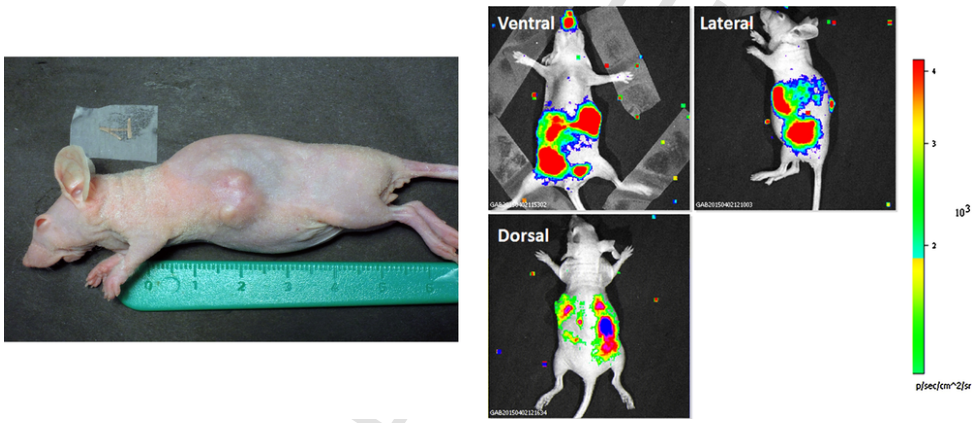

Fig. 4. Assessment of tumor spread by *in vivo* bioluminescence imaging. Photographic image (left) and BLI analysis (right) of a mouse 5 weeks after intra-splenic injection of 1×10^6 HCT 116-fLuc cells.

Cameras, that when maintained at low temperatures can detect photons emitted from an appropriate light source [23]. Tumor cells act as light sources when they express a bioluminescent marker, so 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 are analyzing cells properties like migration, invasion, and proliferation using HCT 116 colorectal cancer stable cell line infected with LV-fLuc to constitutively express luciferase. To generate experimental liver metastasis *in vivo*, cells were administered by intra-splenic injection [24].

Luciferase-expressing cells emit light by oxidation of the substrate luciferin, which can be conveniently administered to the mouse by intra-peritoneal injection. Therefore, by non-invasive *in vivo* bioluminescence imaging (BLI), using a high sensitive Charge-Coupled Device (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 [25]. 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 [26]. 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

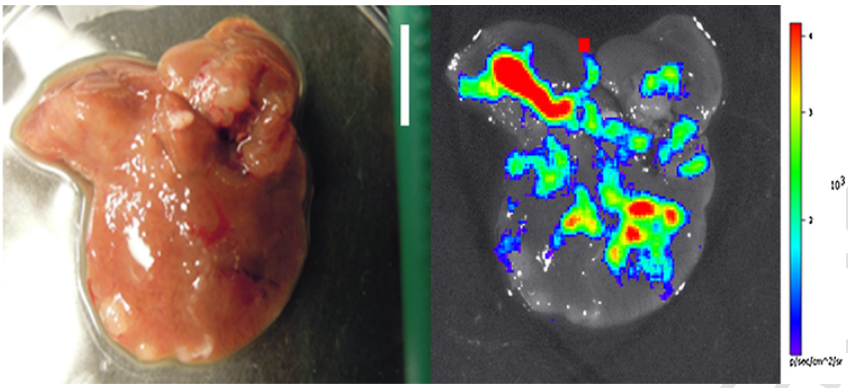

Fig. 5. Assessment of hepatic metastatization by *ex vivo* bioluminescence imaging. Photographic image and BLI analysis of the liver explanted from a mouse 5 weeks after intra-splenic injection of 1×10^6 HCT 116-fLuc cells. Macroscopic liver analysis. Left panel: image of the liver acquired with a digital 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.

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.

Overall, we evaluated whether intra-splenic administration of HCT 116-fLuc cells can be instrumental in the analysis colon cancer progression and for mapping tumor cell dissemination in order 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 Hedgehog inhibitor in the natural history of CC *in vivo*. Our unpublished data, in fact, suggest that modulating the Hedgehog pathway may interfere with the metastatic process of the disease, altering cell proliferation, cell plasticity and glucose/aminoacid metabolism.

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The study protocol was approved by Italian Authorities for animal health and wellness (#DGSAF 0012430-A-19/06/2013).

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