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Engineering of galectin-3 for glycan-binding optical imaging

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ABSTRACT

Galectin-3 (Gal-3) is a multifunctional glycan-binding protein that participates in many pathophysiological events and has been described as a biomarker and potential therapeutic target for severe disorders, such as cancer. Several probes for Gal-3 or its ligands have been developed, however both the pathophysiological mechanisms and potential biomedical applications of Gal-3 remain not fully assessed. Molecular imaging using bioluminescent probes provides great sensitivity for *in vivo* and *in vitro* analysis for both cellular and whole multicellular organism tracking and target detection. Here, we engineered a chimeric molecule consisting of Renilla luciferase fused with mouse Gal-3 (RLuc-mGal-3). RLuc-mGal-3 preparation was highly homogenous, soluble, active, and has molecular mass of 65,870.95 Da. This molecule was able to bind to MKN45 cell surface, property which was inhibited by the reduction of Gal-3 ligands on the cell surface by the overexpression of ST6GalNAc-I. In order to obtain an efficient and stable delivery system, RLuc-mGal-3 was adsorbed to poly-lactic acid nanoparticles, which increased binding to MKN45 cells *in vitro*. Furthermore, bioluminescence imaging showed that RLuc-mGal-3 was able to indicate the presence of implanted tumor in mice, event drastically inhibited by the presence of lactose. This novel bioluminescent chimeric molecule offers a safe and highly sensitive alternative to fluorescent and radiolabeled probes with potential application in biomedical research for a better understanding of the distribution and fate of Gal-3 and its ligands *in vitro* and *in vivo*.

1. Introduction

Galectins are a group of proteins that bind to glycoconjugates containing β-galactosides and share a conserved primary structure homology in their carbohydrate recognition domain (CRD). Galectin-3 (Gal-3), the chimera-type galectin, has ~30 kDa and only one CRD associated to long N-terminal domain [1]. The N-terminal domain of Gal-3 is involved in protein-protein interactions and allows its oligomerization upon binding to multivalent oligosaccharides [2,3]. Gal-3 is ubiquitously express and plays multiple biological functions, depending on its subcellular localization. For example, in the cytoplasm, several molecules have been identified as Gal-3 binding partners, playing a relevant role in apoptotic events [4–6]. Gal-3 participates in various biological events, including cellular homeostasis, cell differentiation, angiogenesis, and immune regulation [7–9].

Indeed, Gal-3 and its ligands have been described as biomarkers of many diseases such as cardiovascular and autoimmune illnesses [10–14]. The expression levels of Gal-3 are often altered in cancer and have been correlated with increased tumor susceptibility, chemotherapeutic drug resistance, aggressiveness and the acquisition of a metastatic phenotype [15–18]. Considering the wide biological functions of Gal-3, future research is still needed to better understand the conditions under which Gal-3 affects several

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pathological conditions, and the role of Gal-3 ligands, which can vary depending on the disorder stages.

The recombinant fusion of Gal-3 with fluorescent and enzymatic probes have gained interest as tools to study the impact of glycobiology of tumor microenvironment [19,20]. Noninvasive optical imaging has been widely used for the real-time detection of molecular targets, biological processes and drug tests with several advantages over other tools: no exposure to ionizing radiation, quantifiability and low cost [21]. Bioluminescent imaging has a very high sensitivity and can be efficiently performed using Renilla, a 36 kDa luciferase widely used a reporter for cell tracking, animal imaging and detection of specific targets [22–24].

Hence, here we developed a suitable new probe, a recombinant mouse Galectin-3 fused with Renilla luciferase (RLuc-mGal-3), associated or not with nanoparticles, to monitor the fate of extracellular and circulating Gal-3 and its ligands *in vitro* and *in vivo* in a gastric cancer model.

2. Material and methods

2.1. Animals

Eight-week-old male Balb/c nude mice were bred and housed at the animal facility of IPEN (Nuclear and Energy Research Institute-Brazil). The experiments were conducted following the relevant laws and were approved by the local animal care committee (protocol number: 181/17).

2.2. Mammalian cell culture

Human gastric cancer cell line (MKN45 cells) transfected with the empty vector pcDNA3.1 (MKN45-Mock) or the full length human ST6GalNAc-I containing vector (MKN45-ST6GalNAc-I) [25] were grown in RPMI supplemented with 10% of fetal bovine serum, 50 μg/mL of gentamicin and 300 μg mL^{-1} of geneticin (Gibco, Life technologies). Lonza Mycoplasma Detection Kit was used to exclude mycoplasma contamination in cultured cells.

2.3. Expression and purification of RLuc-mGal-3

E. coli BL21-DE3 cells were transformed with the pET-28a (+)-RLuc-mGal-3 vector through heat-shock method [27] and RLuc-mGal3 expression was tested under several conditions using IPTG (Isopropyl β-D-1-thiogalactopyranoside – Sigma). RLuc-mGal-3 purification was performed by two consecutive affinity chromatography steps, sepharose-lactose (Sigma) and HisTrap™ (GE Healthcare) columns.

2.4. Hemagglutination assay

The lectin activity of purified RLuc-mGal-3 was determined with an hemagglutination assay using a 96 well round bottom microplates, as described previously [26]. Briefly, RLuc-mGal-3 (3 μmol L^{-1}) was added to mouse erythrocytes suspension in PBS (2%) in the presence or absence of a sugar hapten inhibitor (lactose) or a sugar hapten non-inhibitor (sucrose), at different concentrations (20, 40, 60 and 80 mmol L^{-1}).

2.5. Mass spectrometry (MALDI-TOF/TOF)

RLuc-mGal-3 molecular mass was determined using MALDI-TOF/TOF mass spectrometer (ultrafleXtreme, BrukerDaltonics, Bremen, DE) as previous described [27]. 1 μL of a solution of acetonitrile 30% (TA 30, v:v) and trifluoroacetic acid (TFA) 0.1% saturated with Sinapinic acid matrix was used to dilute (1:1, v:v) the purified RLuc-mGal-3 (1.9 mg/mL).

2.6. Poly-lactic acid nanoparticles (PLA-NPs) containing RLuc-mGal-3

PLA-NPs were made using the double emulsion solvent evaporation method with the poly-lactic acid (PLA) polymer according to previously established protocols [28] were 1% polyvinyl alcohol (PVA) was dripped into 100 μg of RLuc-mGal-3 containing PLA. The mean size and the polydispersity index (PDI) of PLA-NP containing RLuc-mGal-3 (NP-RLuc-mGal-3) were assessed using DLS (Dynamic Light Scattering).

2.7. RLuc-mGal-3 interaction with MKN45 cells

MKN45-Mock and MKN45-ST6GalNAc-I cells were incubated with 1, 10, and 50 μg of RLuc-mGal-3 or NP-RLuc-mGal-3 for 2, 16, and 24 h with or without lactose (100 mmol L^{-1}). Then, 6 nM of ViviRen substrate (Promega Corporation) was added to the microplates (10 min at room temperature) and luminescence was measured using the GloMax® Discover System (promega).

2.8. Assessment of RLuc-mGal-3 in vivo

MKN45 cells (1×10^6) were subcutaneously inoculated in Balb/c mice and tumor growth was followed for 3 weeks. Then, RLuc-mGal-3 (100 μg) in the presence or absence of lactose (100 mmol L^{-1}) was administered intratumorally in MKN45-bearing mice. After 8 h, animals were anesthetized with isoflurane, ViviRen (0.295 mmol L^{-1}) was injected intratumorally and bioluminescence was analyzed for 15 min using the MSFX-Pro equipment and Bruker Molecular Imaging software (Bruker BioSpin Corporation).

2.9. Radio labeling of RLuc-mGal3

RLuc-mGal3 (150 μg) was conjugated with succinimidyl-6-hydrazinopyridine-3-carboxylate (NHS-hynic, Future-Chem) and then, $^{99m}TcO_4$- solution (150 MBq) was added to RLuc-mGal3-HYNIC. The radiochemical purity of RLuc-mGal3-HYNIC-^{99m}Tc was determined by instant thin-layer chromatography-silica gel (ITLC-SG, Agilent).

2.10. MicroSPECT/CT imaging

RLuc-mGal3-HYNIC-^{99m}Tc (37 MBq) was injected intratumorally in MKN45 tumor-bearing mice. After 2 h, SPECT data was recorded followed by a CT scan using the Albira microPET/SPECT/CT imaging system (Bruker Biospin Corporation). Images were reconstructed with Albira software and processed with PMOD software (PMOD Technologies).

2.11. Statistical analysis

The results were expressed as the mean ± SD of at least three independent experiments. Statistical analysis were done using the GraphPad Prism 6.0 software and $p < 0.05$ used as reference for statistical significance.

3. Results

3.1. Production of soluble RLuc-mGal-3

The success of the recombinant RLuc-mGal-3 production and purification, using a sepharose-lactose and nickel resins, was

evaluated by SDS-PAGE where a single band of approximately 66 kDa was detected (Fig. 1). The cloning strategy, electrophoretic analysis and standardization of procedures to obtain the soluble recombinant RLuc-mGal-3 can be found in Supplementary Fig. S1. The capacity of RLuc-mGal-3 binding to sepharose-lactose resin is an evidence that its carbohydrate recognition domain (CRD) is active.

3.2. Molecular mass determination and hemagglutinating activity of RLuc-mGal-3

The RLuc-mGal-3 accurate molecular weight was observed at *m/z* 65,870.95 at MALDI-TOF/MS analysis (Fig. 2A). Purified RLuc-mGal-3 effectively promoted erythrocytes agglutination in a lactose-dependent manner (Fig. 2B), confirming that the lectin property of Gal-3 was preserved (Fig. 1A).

3.3. Cell-binding activity of RLuc-mGal-3

MKN45-ST6GalNAc-I cells presented a significant decrease of RLuc-mGal-3 binding sites compared to MKN45-Mock cells (Fig. 3A), which agrees with previous report [25]. Then, NP-RLuc-mGal-3, with an average size of 188 nm (Fig. 3B and C) and low PDI (0.06) indicating a homogeneous size distribution, was added to MKN45-Mock cells for 2 and 16 h showing increased binding ability to tumors cells when compared to RLuc-mGal-3 (Fig. 3D).

3.4. In vivo binding activity of RLuc-mGal-3

To evaluate RLuc-mGal-3 glycan binding ability to cancer cells *in vivo*, we subsequently intratumorally injected 100 μg of RLuc-mGal-3 in the presence or absence of lactose in MKN45-bearing mice. Eight hours post injection, the Renilla substrate (ViviRen) was intratumorally injected and a robust luminescence signal was detected in the tumor area. Interestingly, lactose was able to reduce the signal dramatically (Fig. 4A). Likewise, the intratumoral injection of RLuc-mGal3 radiolabeled with ^{99m}Tc demonstrated the ability of Gal-3 to bind to the tumor site in a carbohydrate dependent manner (Supplementary Fig. S2 and Fig. 4B and C). Altogether our data indicate that RLuc-mGal3 can be a valuable tool to study the role of galectin-3 *in vivo*.

4. Discussion

Galectin-3 is a multifaceted glycan-binding protein whose biological functions and biomedical applications make this molecule a target for basic/clinical investigations and for the development of theranostic tools. In this context, the use of Galectin-3 probes, for molecular imaging approaches have gained great interest and attention by the scientific community. Bioluminescence provides a substantial advantage over fluorescence and radiolabeled probes which are widely used within living cells or organism because of the extreme specificity of its signal, low-cost and the absence of ionizing radiation. However, the engineering of recombinant proteins with Luciferase or Renilla, still poses a biotechnological challenge. In this work we generated a novel recombinant bioluminescent chimeric galectin-3 (RLuc-mGal-3), in a soluble or adsorbed to nanoparticles form, which showed a high capacity to bind *in vitro* and *in vivo* cancer cells.

RLuc-mGal-3 was expressed as a soluble and active molecule in the cytoplasm of the well-established strain *E. coli* BL21 (DE3) [29]. Cytoplasmic expression is fast, simple and provides a huge amount of protein, making this system inexpensive and reproducible [30]. An homogeneous preparation of RLuc-mGal-3 could be achieved after a double-affinity chromatography purification, using both the carbohydrate binding activity [31] and nickel affinity chromatography through hexa-histidine tag, mainly used for RLuc fused proteins purification [32].

Gal-3 is present as a monomer in solution and can form oligomers in a concentration dependent manner through its N-terminal domain [3,33,34]. In this work, the chimeric RLuc-mGal-3 was intentionally designed to preserve Gal-3 CRD in order to exploit the lectin properties of Gal-3. Interestingly, RLuc-mGal-3 exhibited hemagglutinating activity through a sensitive-lactose manner, even though its N-terminal domain was fused to Renilla luciferase. This data suggests that RLuc-mGal-3 is able to form oligomers in solution, since we have tested RLuc-mGal-3 concentrations greater than 20 μg mL^{-1}, a critical concentration for Gal-3 self-association [34]. Others galectin-3 chimeric molecules showed preservation of their lectin activities regardless of their oligomeric state [19]. Ochieng and co-workers 1998 [35] described that the Pro-Gly-Tyr motifs, located between N- and C-terminal, are associated with Gal-3 oligomerization. Considering these observations, we suggest that RLuc-mGal-3 was able to induce hemagglutination due to the fact that Pro-Gly-Tyr motifs could interact among molecules, forming oligomers, and the RLuc portion did not cause steric hindrance.

The Gal-3 chimeric molecules reported so far did not lose their ability to bind to their targets [19] and therefore, its use have been explored by several researchers. For example, Gal-3 has been expressed as a fusion-protein using green fluorescent protein [36] and alkaline phosphatase [20] to investigate its tissue distribution and binding patterns in several biological scenarios. Still, to date, no reports have been found on the cloning and expression of recombinant soluble and active forms of Gal-3 fused to a bioluminescent molecules such as Renilla luciferase, an enzyme which catalyzes the oxidation of coelenterazine, generating a blue light of 480 nm [37].

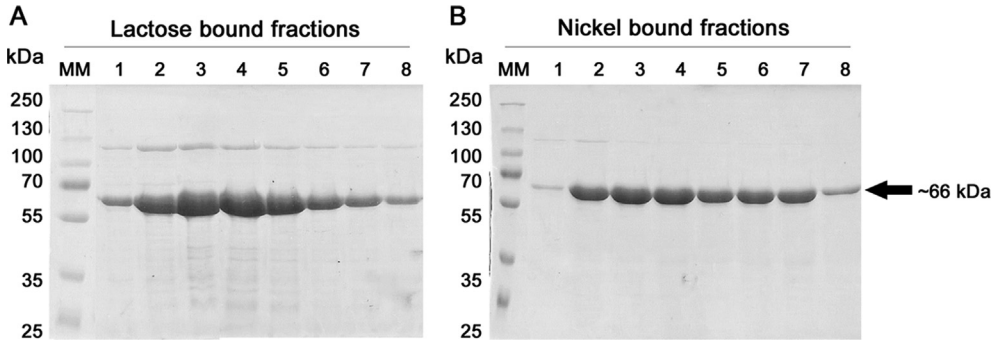

Fig. 1. SDS-PAGE of RLuc-mGal3 two-step-purification procedure. (A) Lactose affinity. Lanes 1 to 8 collected samples from 100 mM lactose elution. (B) Nickel affinity. Lanes 1 to 4 collected samples from 150 mmol L^{-1} imidazole elution; lanes 5 to 8, collected samples from 250 mmol L^{-1} imidazole elution. MM, protein molecular weight markers.

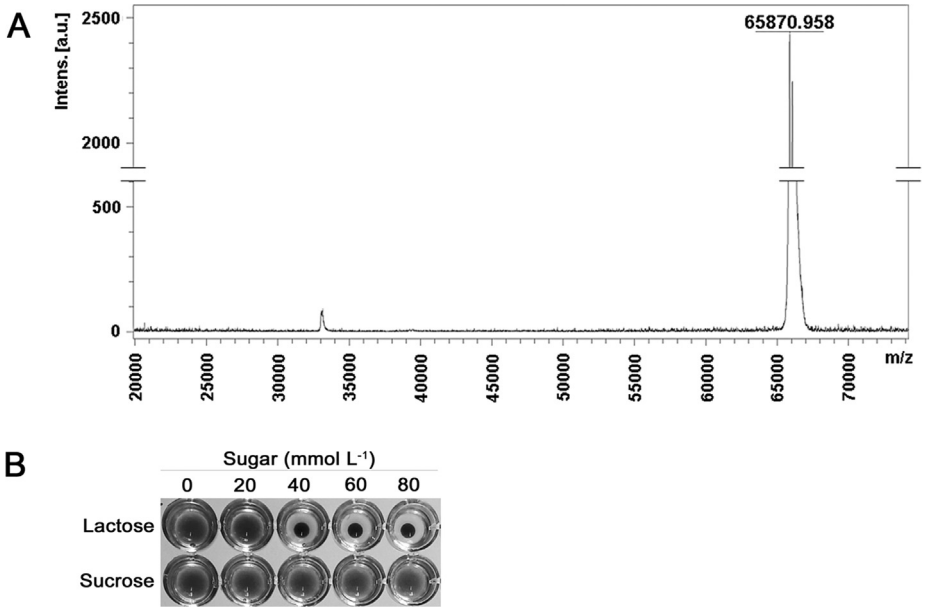

Fig. 2. MALDI-TOF/MS and hemagglutination analysis of purified RLuc-mGal-3. (A) Recombinant RLuc-mGal-3 exhibited a molecular weight 65,870.95 Da. (B) RLuc-mGal-3 (3 μmol L^{-1}) was incubated with different concentrations of lactose and sucrose (20, 40, 60, and 80 mmol L^{-1}) and mouse red blood cells a final concentration of 2%.

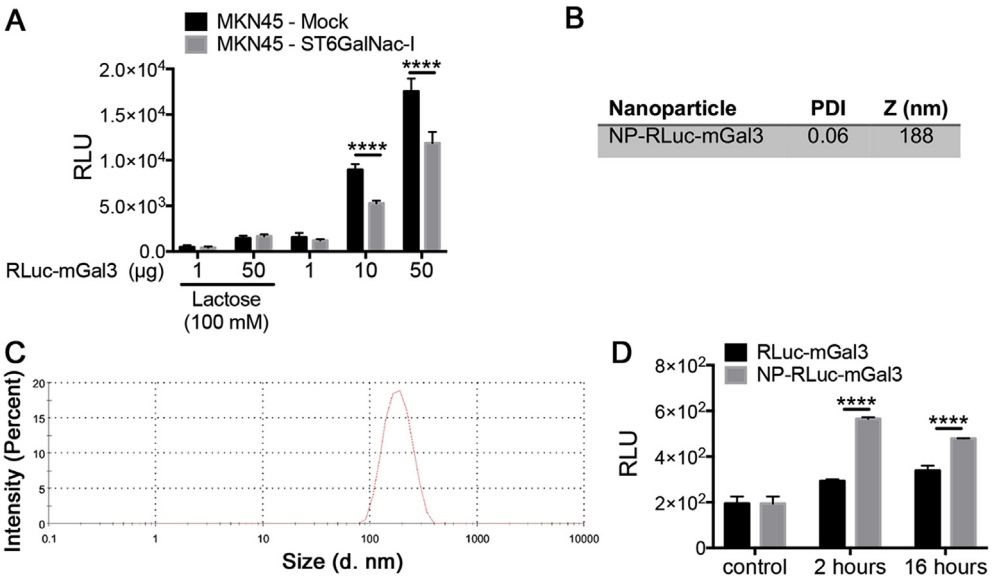

Fig. 3. Cell-binding activity of RLuc-mGal-3. (A) 2×10^3 MKN45-Mock or MKN45-ST6GalNAc-I were incubated with 1, 10 and 50 μg of RLuc-mGal-3 in the presence or absence of lactose (100 mmol L^{-1}). ViviRen substrate (6 nM) was added to each well and the luminescence was read. (B) Polydispersity index PDI and diameter (Z) values of NP-RLuc-mGal-3. (C) NP-RLuc-mGal-3 size distribution. (D) 2×10^3 MKN45-Mock and MKN45-ST6GalNAc-I were incubated with 10 μg of RLuc-mGal-3 or NP-RLuc-mGal-3 for 2 or 16 h. ViviRen substrate (6 nM) was added to each well and the luminescence was read. A and D - Results are expressed as the mean RLU (relative luciferase units) ± SD, ****$p < 0.0001$.

The absence of mammalian endogenous luciferases makes this molecule a suitable probe for *in vitro* and *in vivo* cell assays [38]. Many authors have been taking advantage of the low cost, high sensitivity and easy manipulation that characterizes bioluminescence as a suitable choice for *in vivo* and *in vitro* molecular imaging [21,32,39].

Based on data described by Lee and colleagues [40], we used the nanotechnology approach to improve the capacity of RLuc-mGal-3 binding on cell surface, consequently enhancing the signal ratio and sensitivity of this probe. Thus, we prepared poly-lactic acid nanoparticles conjugated with RLuc-mGal-3 which exhibited greater binding capacity to tumor gastric cells than RLuc-mGal-3 in solution, possibly due to the higher number of exposed RLuc-mGal-3 at nanoparticles surface as reported using antibodies [40].

It is well-known that Gal-3 is involved in several pathological conditions and Gal-3-ligand inhibitors can prevent unwanted events promoted by Gal-3 [41–43]. For instance, Gal-3 and its binding pairs (Gal-3bp) play critical roles in the development of venous thrombosis (VT) and elevated circulating Gal-3bp is associated with acute VT [44]. Another research group analyzed, comparatively, bronchoalveolar lavage fluid from asthmatics and healthy patients and detected different profiles of Gal-3 and Gal-8 ligands correlated with pathophysiological parameters of asthma [45]. Then, we suggest that unhealthy conditions associated with

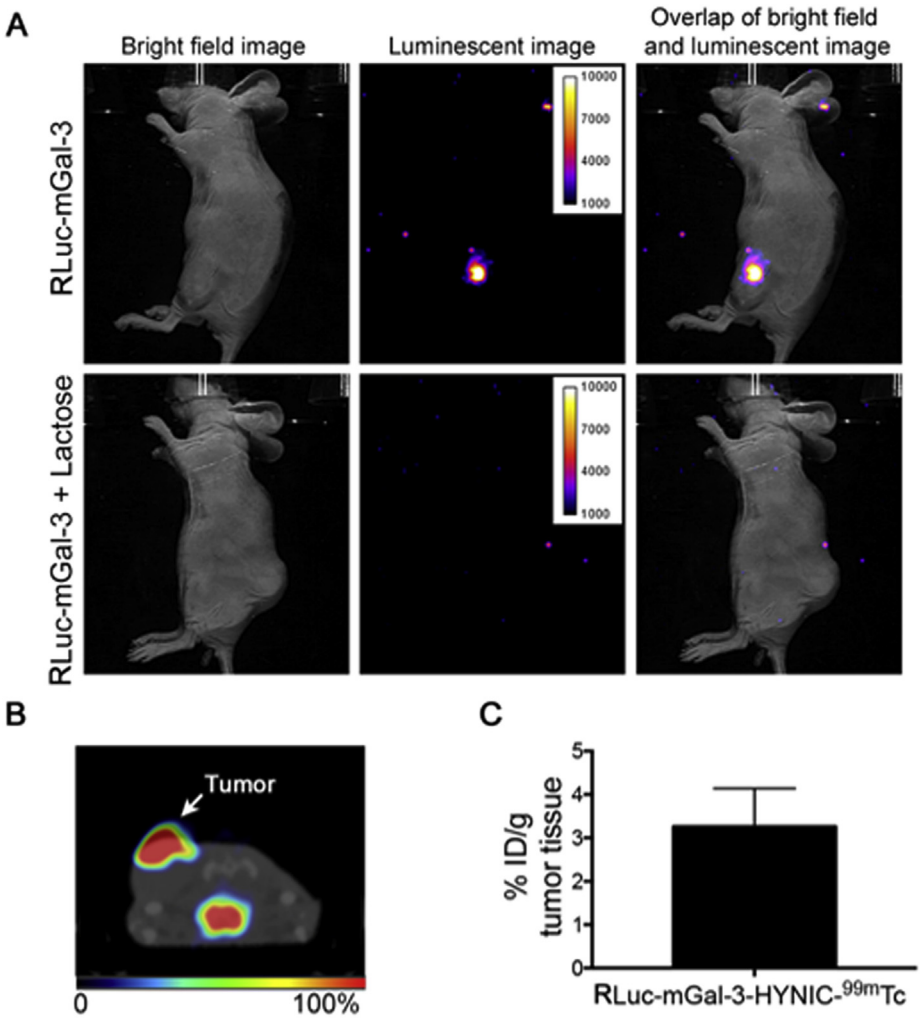

Fig. 4. RLuc-mGal3 binds to tumor *in vivo* in a carbohydrate-dependent manner. (A) Luminescent image of MKN45 tumor-bearing mice 8 h after the intratumoral injection of RLuc-mGal-3 (100 μg) in the presence or absence of lactose (100 mM). Images were taken immediately after the intratumoral administration of Renilla substrate (ViviRen) and were acquired for 15 min at the left side position. (B) Contiguous transaxial SPECT/CT section of MKN45 xenografts bearing mice 2 h after RLuc-mGal3-HYNIC-^{99m}Tc intratumoral injection. (C) The mean value of RLuc-mGal3-HYNIC-^{99m}Tc uptake by the tumor 2 h after intratumoral injection.

differential expression of Gal-3 ligands can be non-invasively, *in vitro* and *in vivo,* assessed by RLuc-mGal-3, including cancer. In addition, our findings can be used as basis for future studies on the development of potential Gal-3 inhibitors. In this work, we analyzed whether the interaction between RLuc-mGal-3 and cancer cells occurs in a carbohydrate recognition manner. For this purpose, we performed inhibition assays using an specific hapten-sugar (lactose) or ST6GalNAc-I-overexpressing gastric cancer cells which expressed lower levels of Gal-3 carbohydrate ligands [25]. As expected, both strategies showed the involvement of Gal-3 CRD on the cell cancer recognition by RLuc-mGal-3. The intratumoral injection of RLuc-mGal3 and RLuc-mGal3-^{99m}Tc showed that Gal-3 is able to bind *in vivo* to the microenvironmental tumor site in a carbohydrate dependent manner. These data suggested that RLuc-mGal3 could be a promising tool to study the glycobiology of several illnesses *in vivo*.

In summary, we generated a novel recombinant chimeric protein, RLuc-mGal-3, which exhibit lectin and bioluminescent features. This molecule showed a high capacity to bind cancer gastric cells MKN45, which was enhanced by coupling RLuc-mGal-3 to Poly-lactic acid nanoparticles. Also, RLuc-mGal-3 recognizes galectin binding pairs on tumor surface using mouse model of gastric cancer. Based on these results, we consider that RLuc-mGal-3 might be a new suitable tool applicable in non-invasive studies related to Gal-3 fate and binding pairs in many pathophysiologic conditions.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.10.161.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.10.161.

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