Original Article: Laboratory Investigation

Glypican-1 as a target for fluorescence molecular imaging of bladder cancer

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Running title: Imaging bladder cancer by Miltuximab®-IR800

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Abstract

Objectives: To investigate whether anti-Glypican-1 antibody Miltuximab® conjugated with near-infrared dye IRDye800CW can be used for in vivo fluorescence imaging of urothelial carcinoma.

Methods:

The conjugate Miltuximab®-IRDye800CW was produced and characterized by size exclusion chromatography and flow cytometry with Glypican-1-expressing cells. Balb/c Nude mice bearing subcutaneous urothelial carcinoma xenografts were intravenously injected with Miltuximab®-IRDye800CW or control IgG-IRDye800CW and imaged daily by fluorescence

imaging. After ten days, tumors and major organs were collected for ex vivo study of the conjugate biodistribution, including its accumulation in the tumor.

Results:

The intravenous injection of Miltuximab®-IRDye800CW to tumor-bearing mice exhibited its specific accumulation in the tumors with the tumor-to-background ratio of 12.7 ± 2.4, which was significantly higher than that in the control group (4.6 ± 0.9, $P < 0.005$). The ex vivo imaging was consistent with the in vivo findings with tumors from the mice injected with Miltuximab®-IRDye800CW being significantly brighter than the organs or the control tumors.

Conclusions:

The highly specific accumulation and retention of Miltuximab®-IRDye800CW in Glypican-1-expressing tumors in vivo shows its high potential for fluorescence imaging of urothelial carcinoma and warrants its further investigation towards clinical translation.

Keywords: Urinary Bladder Neoplasms, Fluorescence-Guided Surgery, Glypican-1, Monoclonal Antibodies, Molecular Imaging

1. Introduction

Approximately 75% of bladder cancer patients present with non-muscle-invasive urothelial carcinoma (UC) with tumors confined to the urothelium and subepithelial layer (1). Such tumors are usually removed by transurethral endoscopic resection followed by intravesical immunotherapy or chemotherapy and regular surveillance cystoscopies (2). While this treatment is often effective, up to 50% of patients eventually suffer recurrence or invasive progression which may require cystectomy and systemic therapy (3). The high rate of recurrence and long-term surveillance make bladder cancer a major health issue and economic burden (4). An incomplete initial resection has been hypothesized to contribute to the high rates of recurrence and progression of UC (5–7).

Fluorescence cystoscopy, using hexaminolevulinate to improve completeness of initial resection, is one recommended option, however, it suffers from low specificity, limited contrast, and the need for intravesical instillation of the drug 1 - 2 h before cystoscopy, with retention of the drug until the procedure (2). Orally administered 5-ALA solves the problem of intravesical instillation, however it shares the other shortfalls of hexaminolevulinate including the need for careful timing between the administration and cystoscopy (8). Near-infrared (NIR) fluorescent molecular imaging has potential to overcome the limitations of hexaminolevulinate (9). Such imaging usually involves the use of a conjugate of a monoclonal antibody with a NIR fluorescent dye, where the antibody and the dye provide optimal specificity and imaging performance, respectively (9,10). Provided the imaging antibody-dye conjugate is safe and

specific, it can be administered intravenously, eliminating the need for intravesical administration and retention of the drug before cystoscopy. The intravenous administration of an antibody-dye conjugates for fluorescence-guided surgery has been studied extensively and successfully used in clinical trials in breast and brain cancer patients (11–14).

Recently introduced NIR dye IRDye800CW (IR800) appears one of the most promising candidates for intra-operative molecular imaging (13). While several anti-EGFR monoclonal antibodies coupled to IR800 have been successfully validated in clinical trials (11–14), the notoriously high antigenic heterogeneity of UC requires expansion of the existing suite of antibodies specific to this disease for highly sensitive and specific imaging (15). Glypican-1 is a novel oncotarget, detected in UC and a range of other solid tumors, but not in normal adult tissue (16–23). We hypothesized that clinical stage anti-Glypican-1 antibody Miltuximab® (24), conjugated with IR800 may be suitable for fluorescence molecular imaging of UC. In this study, we report the evaluation of Miltuximab®-IR800 for fluorescent molecular imaging of UC in a mouse xenograft model after its intravenous administration.

2. Materials and methods

2.1. Cell lines

Urothelial carcinoma cell lines were cultured in a 5% CO_2 tissue culture incubator at 37 ºC following standard protocols in recommended media supplemented with 10% heat inactivated (56 º C, 30 min) Fetal Bovine Serum (FBS; Scientifix, New Zealand). For subculturing, the cells were detached by incubation for 20 min in 2 mM ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS) at 37 ºC after a wash with 1X PBS.

2.2. Characterization of Glypican-1 expression in urothelial carcinoma

Analysis of the GPC-1 expression in published microarray data sets was performed using the Oncomine (www.oncomine.org) concept analysis tool (25). Glypican-1 expression at the RNA level was analyzed in UC cell lines (T24, RT4, UM-UC-3, UM-UC-6, UM-UC-13, UM-UC14, UM-UC-9, 5637, 253J, J82, UM-UC-10, UM-UC-12) and established patient-derived cells (**BC8149 & **BC8447). Cultured cells were lysed in Trizol (Life Technologies, USA). RNA extraction from Trizol was performed according to the manufacturer's protocol; BioRad iScript cDNA synthesis kit was used for reverse transcriptase polymerase chain reaction (PCR) (BioRad, USA). RNA from BC8149 and BC8447 was kindly provided by Dr. Philip Palmbos (UM). Quantitative PCR was performed using BioRAD SYBR Green Mastermix on an Applied Biosystems 7300 Real-Time PCR system. All reactions were performed in triplicates.

Fold mRNA expression was calculated using $2^{-\Delta\Delta CT}$ method (26). Human GPC-1 primers were purchased from Sino Biological (Cat # HP100583; USA).

At the protein level, we characterized the GPC-1 expression in UC by immunohistochemistry, western blot, and flow cytometry. Immunohistochemistry was performed using tissue microarrays with single cores of 51 UC from the UMRCC (University of Michigan Rogel Cancer Center) Tissue and Molecular Pathology Core. Staining for Glypican-1 was performed on an autostainer (DAKO, USA). Stained slides were interpreted as positive when more than 5% of tumor cells demonstrated strong reactivity with the antibody. Western blot was performed as previously described (27). The quantification of GPC-1 in UC cells was performed by flow cytometry using anti-GPC-1 antibody MIL-38 (Glytherix Ltd. Australia), a murine version of Miltuximab®, and a QIFIKIT (Quantitative Immunofluorescence Intensity kit, Dako) following the manufacturer's protocol.

2.3. Conjugation of Miltuximab® with IR800 and its characterization

Miltuximab® (Glytherix Ltd., Australia) was conjugated with the N-hydroxysuccinimide (NHS) ester of the NIR fluorescent dye IR800 (for lysine binding on the antibody) using an IRDye 800CW Protein Labelling Kit – High MW (928-33040, Li-Cor Biosciences, USA) as per the manufacturer's protocol. The Miltuximab®-IR800 conjugate was subsequently separated from free dye using a centrifugal column. To determine the final concentration of the conjugate and the dye/antibody molar ratio, absorbance spectra at 280 nm and 780 nm were determined by a microplate reader Pherastar (BMG Labtech, Germany). As a control, a human IgG Isotype Control antibody (cat # 31154, Thermo Fisher Scientific, USA) was conjugated with IR800 and characterized using the same method. The purities of the antibody and conjugates were analyzed by size exclusion chromatography high performance liquid chromatography (SEC-HPLC) on an Agilent Bio SEC-3 column with UV detection at 280 nm, flow rate of 0.3 mL/min and column temperature of 25 ºC. A control protein standard mixture (Bio-Rad, USA) was run prior to analysis of each sample. The binding of Miltuximab®-IR800 to UC-6 cells in vitro was compared to that of unconjugated Miltuximab® and control IgG-IR800 to the same cell line by flow cytometry following previously published protocol (20).

2.4. Establishment of subcutaneous xenografts

The work with animals was performed in accordance with the Australian code for the care and use of animals for scientific purposes and approved by Macquarie University Animal Ethics Committee (AEC Reference No.: 2018/015). UC-6 cells in serum-free media with Matrigel Basement Membrane matrix (Corning, USA) were subcutaneously inoculated to 10 8-week-old Balb/c nude female mice (Animal Resource Centre, Australia) anaesthetized by 2%

isoflurane inhalation via a nose cone. The mice were monitored for signs of distress and weighted daily, while the tumor growth was monitored twice weekly by caliper measurement.

2.5. Intravenous administration of Miltuximab®-IR800 and in vivo imaging

When the tumors reached 1000 mm^3 in volume, 8 mice with tumors of equivalent size were selected, randomized into 2 groups of 4 and intravenously injected with 6 mg/kg of Miltuximab®-IR800 or the control IgG-IR800. For the injection, the mice were illuminated by a heating lamp for 10 min and anaesthetized by 5% isoflurane inhalation via a nose cone. The injection was then performed into the tail vein using a 1 mL syringe with a 30G needle, followed by supervised recovery.

After the intravenous administration of the conjugate, the mice were daily monitored for signs of toxicity and imaged daily by a fluorescence imager Odyssey CLx (Li-Cor Biosciences, USA) at 3 h, 10 h, 24 h post-injection, and then daily for ten days. The image was performed in Image Studio software (Li-Cor Biosciences, USA). Regions of interest of equal size were placed on the tumor and on the mouse back away from the tumor. The total fluorescence intensity of these regions was then used to calculate the tumor-to-background ratio (TBR).

2.6. Ex vivo study of the biodistribution of Miltuximab®-IR800

Ten days after the conjugate administration, the tumor and major organs were collected from the mice and imaged by the Odyssey CLx imager. To assess the distribution of the conjugate within the tumor, 5 µm sections were prepared and studied by the Odyssey CLx imager. By using Image Studio software, each organ or tumor section were selected as a region of interest and the mean fluorescence intensity (MFI) of these regions was compared.

2.7. Statistical analysis

The values are expressed as mean ± standard error of the mean (SEM). The statistical analyses were performed using GraphPad Prism 8.3.0 software. The in vivo TBR and ex vivo MFI were compared between the groups using the unpaired student's t-test with Welch's correction. A P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Glypican-1 expression in urothelial carcinoma

We confirmed GPC-1 overexpression in infiltrating UC at the RNA level using public database (Oncomine) (Fig. 1, A) and qPCR performed on cell lines and patient derived cells (Fig.1, B). The GPC-1 overexpression at the protein level was confirmed by positive immunohistochemical staining in high-grade invasive UC tissue samples from a tissue microarray (Fig. 1, C) and by western blot in UC cell lines 5637, UC-14, UC-9, UC-6, UC-13,

UC-3 and high-grade infiltrating patient-derived UC cells (**BC8149 & **BC8447) using prostate cancer cell lines LNCaP and DU-145 as a negative and positive controls, respectively (Fig 1, D). Additionally, we carried out the flow cytometry assaying and quantified the expression of GPC-1 in UC cell lines T-24 and UC-6 (Fig. S1.).

3.2. Characterization of Miltuximab®-IR800

An NHS ester of the NIR fluorescent dye IR800 was conjugated with Miltuximab® or a control isotype human IgG_1 antibody. The final concentration was found to be 0.57 mg/mL and the average dye-to-protein molecular ratio was approximately 1:1 in both conjugates. Miltuximab®-IR800 was subsequently characterized by SEC-HPLC and compared to unconjugated Miltuximab®. The Miltuximab®-IR800 was found to be 81.3 ± 1.3 % monomeric, which was lower than 90.2 ± 0.1 % for unconjugated Miltuximab®. Such reduction of the monomeric fraction is consistent with approximately 90% of Miltuximab® molecules being labelled by IR800. SEC-HPLC has also shown an increase in the retention time of the conjugate, in line with an increase of molecular weight.

The flow cytometry analysis confirmed that the conjugation did not affect the binding of Miltuximab®-IR800 to UC cells UC-6 (98.93% ± 1.33% positive; MFI = 12,273 ± 622 a.u.), which was not significantly different from that of unconjugated Miltuximab® (99.83% ± 0.08% positive; MFI = 10,166 ± 2,726 a.u.) (Fig. 2, A). As shown in Figure 2, B, flow cytometry also confirmed NIR fluorescence of Miltuximab®-IR800 (MFI = 543.3 ± 11.9 a.u.) and lack of binding of the control conjugate IgG-IR800 to UC-6 cells (MFI = 23.0 ± 0.6 a.u.).

3.3. In vivo fluorescence imaging

To assess the specific tumor accumulation of Miltuximab®-IR800, we prepared a group of 8 mice inoculated with UC-6 cells on the hind limb. The group was split into 4 tested and 4 control animals and intravenously injected with Miltuximab®-IR800 or IgG-IR800 as a control for passive accumulation. The mice were imaged in vivo at 3 h, 24 h and then daily for 10 days using a fluorescence imager, (Fig. 3 A and B). As shown in Figure 3 B, the TBR (contrast) exhibited persistent growth over the observation period. While the TBR was comparable between the groups on Day 1 (Miltuximab®-IR800 TBR = 2.2 ± 0.23; IgG-IR800 TBR = 1.9 ± 0.16), by Day 10, the decreasing background fluorescence (Fig. 3, C and D) resulted in the contrast increase. This trend was especially prominent in the animals administered with Miltuximab®-IR800. TBR of this group reached 12.7 ± 2.35 and was significantly greater than that of the control group (4.6 ± 0.91, $P < 0.005$).

3.4. Biodistribution of systemically administered Miltuximab®-IR800

The laboratory animals were sacrificed on Day 10 after the injection of Miltuximab®-IR800, and their tumors and major organs were collected for ex vivo fluorescence imaging. Panels A and B of Figure 4 show that the tumors of the mice injected with Miltuximab®-IR800 exhibited greater uptake of IR800 measured in terms of the fluorescence intensity, MFI = 1082 ± 298 a.u. MFI in the tumors was greater than that in the organs (MFI = 159 ± 71 a.u.) or tumors extracted from the control mice (MFI = 540 ± 113 a.u.). Injection of Miltuximab®-IR800 resulted in the higher tumor-to-liver ratio than that of the control group (6.0 ± 1.3 versus 2.2 ± 0.1, respectively). This result speaks in favor of the specificity of the tumor targeting by Miltuximab®-IR800. Also, in line with the specific accumulation of Miltuximab®-IR800 in the tumor and less unbound conjugate in circulation, the fluorescence of the skin, spleen, and heart was lower in these mice compared to the control group.

The tumors were subsequently sectioned and reimaged to avoid artefacts of whole-organ imaging and better assess the sensitivity of the imaging with Miltuximab®-IR800. Panels C and D, Figure 4, show that 5-μm tumor sections from the mice injected with Miltuximab®-IR800 produced approximately two-times-stronger fluorescence (MFI = 52.1 ± 9.7 a.u.) than that of the tumor sections of the same thickness from the control mice (MFI = 23.7 ± 6.1 a.u.). These findings were consistent with the assessment of the specific and non-specific tumor uptake in the whole-mouse in vivo and whole-organ ex vivo imaging and support specific accumulation of Miltuximab®-IR800 in the tumor.

4. Discussion

We investigated the application of fluorescent immunoconjugate Miltuximab®-IR800 for molecular imaging of UC. Firstly, we confirmed overexpression of GPC-1 in UC but not normal urothelium on genetic and protein levels using genetic database, cell lines and tissue microarrays. We then conjugated Miltuximab® with IR800 and confirmed that its binding to GPC-1-expressing cells is comparable to that of unconjugated Miltuximab®. The accumulation of Miltuximab®-IR800 in tumors in vivo was established using subcutaneous UC xenografts and systemic administration of the tested immunoconjugate. We found that Miltuximab®-IR800 was successfully delivered to the tumors producing high fluorescence contrast, with contrast increasing over 10 days. The results of the ex vivo investigation of the animal tumors and organs corroborated the in vivo findings and confirmed the specific accumulation and retention of Miltuximab®-IR800 in the tumors. Bright fluorescence of 5-μm tumor sections indicated high

sensitivity of this technique, which is crucial for the thorough detection of the residual pathology after resection.

The high specificity of Miltuximab®-IR800 was demonstrated by the lower TBR in the mice treated with the control conjugate IgG-IR800. The low-level tumor fluorescence caused by IgG-IR800 was likely due to passive accumulation and enhanced permeability and retention (EPR) effect of tumors (28), however, it was far inferior to that of Miltuximab®-IR800. Compared to the control group, the mice injected with Miltuximab®-IR800 had two-times-higher fluorescence of the tumor and, importantly, approximately three-times-higher tumor-to-liver ratio, which further supports high specificity of the tumor accumulation of Miltuximab®-IR800. Due to the washout of the unbound conjugate from normal tissue, and its retention in the tumor, the TBR reached 2.2 allowing tumour visualization as early as 24h after the injection and continued to grow throughout the following 10-day period, an important characteristic for an imaging agent that can be delivered days prior to imaging in a clinical setting. Importantly, while some heterogeneity in fluorescence can be appreciated in tumor sections due to a combination of cancer cells and connective tissue, fluorescence could be detected throughout the tumor (Fig. 4), which is critical for the detection of tumor margins during guided resection and supports the potential utility of Miltuximab®-IR800 for this purpose.

A number of imaging agents have been previously developed for intravesical application. While intravesical delivery may appear advantageous because of the reduced systemic access of drugs (29), it is associated with practical difficulties, such as catheterization of the bladder before the procedure and requiring the patient to retain the drug in the bladder. Moreover, recently, Pan and colleagues have identified that intravesically instilled quantum dots entered the circulation with ensuing accumulation in other organs, potentially mediating off target effects (29). In contrast to the previous studies, we aimed to develop a conjugate that could be administered intravenously to mitigate the need for bladder catheterization and improve access to the deeper tumor layers (30,31).

Based on our work and existing data, Miltuximab®-IR800 has a potential to aid in detection of small flat tumours and non-resected malignant tissue at tumour margins during cystoscopy. It could be administered intravenously prior to cystoscopy and detected by intraoperative fluorescence imaging. The next stage of our work will validate Miltuximab®-IR800 in an orthotopic model of UC. It will allow us to perform a head-to-head comparison of the intravesical and intravenous administration of Miltuximab®-IR800 and elucidate its performance in accessing and marking tumor margins. Apart from the tumor imaging performance, such study would involve assessment of the differences in the biodistribution of the conjugate after systemic or topical administration. To increase the translational relevance of

our future work, we intend to validate Miltuximab®-IR800-assisted tumor detection by using an open-field clinical fluorescence-imaging device. In addition to fluorescence cystoscopy, other applications of Miltuximab®-IR800, such as intraoperative visualisation of metastatic disease in lymph nodes and other organs, or fluorescence urine cytology could be investigated in the future.

While a comprehensive assessment of the safety of Miltuximab®-IR800 will be critical for clinical translation, existing data on safety of Miltuximab® and IR800-antibody conjugates is excellent. In addition to the safety demonstrated by anti-GPC-1 antibodies in mice at up to 50mg/kg (18). , the safety of radio-labelled Miltuximab® was recently shown in a clinical trial (24). The safety of IR800-antibody conjugates has been investigated even more extensively and shown in multiple animal models and clinical trials (13,32–38).

In conclusion, this study for the first time identified GPC-1 as a target for molecular imaging of UC and reported the experimental use of Miltuximab®-IR800 in laboratory animals. The proof-of-concept work described herein shows excellent targeting and retention to the tumor and its high-contrast visualization when Miltuximab®-IR800 is delivered intravenously. Together with existing data on Miltuximab® and IR800-based immunoconjugates, our findings highlight the potential of Miltuximab®-IR800 as an agent for fluorescence molecular imaging of UC.

Acknowledgments

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Conflicts of interest

DHC, AW, MEL, YL and BJW are employed by Glytherix Ltd. ABZ and GSP received grants and other funding with Glytherix Ltd.

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Figure legends

Fig. 1. Expression of GPC-1 in UC tissues, cell lines and tissue microarrays. (A) cDNA microarray data analysis from public database (Oncomine) confirmed GPC-1 overexpression in samples from patients with UC. **(B)** qPCR was performed for GPC-1 expression in a panel of UC cell lines and established patient-derived cells (**BC8149 & **BC8447). **(C)** Immunohistochemical staining of GPC-1 of UC tissue microarray sections. **(D)** GPC-1 protein expression in UC cell lines analyzed by western blot with prostate cancer cell lines LNCaP and DU-145 as a negative and positive controls, respectively, and Actin as a loading control.

Fig. 2. Flow cytometry analysis of the binding of Miltuximab®-IR800 to UC-6 cells compared to unconjugated Miltuximab® (A) or IgG-IR800 (B). (A): The light grey filled histogram represents unstained cells, dashed black line shows the cells incubated only with secondary antibody, solid grey line shows the cells incubated with unconjugated Miltuximab®, and solid black line shows the cells labelled by Miltuximab®-IR800. **(B)**: The light grey filled histogram represents unstained cells, solid grey line shows the cells incubated with IgG-IR800, solid black line shows the cells labelled by Miltuximab®-IR800.

Fig. 3. Imaging and analysis of in vivo biodistribution of Miltuximab®-IR800 or IgG-IR800 in tumor-bearing and control mice. (A) Representative false-color images of the mice injected with Miltuximab®-IR800 or IgG-IR800 at 3 h, 2, 6, and 10 days after intravenous injection with constant imaging parameters and Min/Max pixel values. White arrow points at the tumor. **(B)** The line graph showing tumor-to-background ratio in mice injected with Miltuximab®-IR800 or IgG-IR800. **(C and B)** The bar charts below show the total fluorescence intensity from the regions of interest of equal size placed on the tumor or the back of the mice injected with Miltuximab®-IR800 **(C)** or IgG-IR800 **(D)**. Data are means ± SEM, n = 4, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 4. Ex vivo assessment of the biodistribution of Miltuximab®-IR800. (A) Ex vivo fluorescence imaging of UC-6 tumors and major organs removed from the mice on Day 10 after the intravenous injection of Miltuximab®-IR800 (left) or IgG-IR800 (right). **(B)** Fluorescence of the whole tumors and organs from ex vivo imaging. **(C)** Representative sections of the tumors removed from the mice injected with Miltuximab®-IR800 (left) or IgG-IR800 (right) and **(D)** their fluorescence intensity.

List of supporting information

Supplementary Fig. 1. Histograms of the flow cytometry assays of GPC-1 expression in UC cell lines T-24 and UC-6, as well as lymphoma cell line Raji used as a negative control for the GPC-1 expression.