(A) α -PAP2a-CMDM-Cy5.5

(B) α -PAP2a-Cy5.5 adminitration

(C) α -PAP2a-CMDM-Cy5.5 administration

The specific detection of target tumors by mAb-conjugated SPIO using clinical 1.5-Tesla MRI. Our observations provide clues for reliable active targeting.

Evaluation of Selective Tumor Detection by Clinical Magnetic Resonance Imaging using Antibody-conjugated Superparamagnetic Iron Oxide

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Number of figures: 3; **tables:** 2; **Supporting Information:** 6 figures and supporting methods.

Running title: Specific Tumor Targeting using Clinical MRI

Abstract

Active targeting by monoclonal antibodies (mAbs) combined with nanosize superparamagnetic iron oxide (SPIO) is a promising technology for magnetic resonance imaging (MRI) diagnosis. However, the clinical applicability of this technology has not been investigated using appropriate controls. It is important to evaluate the targeting technology using widely used clinical 1.5-Tesla MRI in addition to the high-Tesla experimental MRI. In this study, we measured mAb-conjugated dextran-coated SPIO nanoparticles (CMDM) *in vivo* using clinical 1.5-Tesla MRI. MRI of tumor-bearing mice was performed using a simple comparison between positive and negative tumors derived from the same genetic background in each mouse. The system provided significant tumor-targeting specificity of the target tumor. To the best of our knowledge, this is the first report on the specific detection of target tumors by mAb-conjugated SPIO using clinical 1.5-Tesla MRI. Our observations provide clues for reliable active targeting using mAb-conjugated SPIO in clinical applications.

Key words: clinical 1.5T-MRI, CMDM, SPIO, monoclonal antibodies, tumor-targeting

1. Introduction

Recent advances in nanotechnology have resulted in the development of various nanosize devices applicable to cancer diagnosis and therapy [1]. Notably, nanoparticles can now be equipped with multifunctional groups suitable for magnetic resonance imaging (MRI) or manipulated drug release [2-6]. These nanodevices, when combined with specific tumor ligands, are potentially powerful tools for safe cancer therapy [6-9]. Although studies have shown that the combined application of nanoparticles with monoclonal antibodies (mAbs) is a promising technology [6, 8-10], their tumor-targeting specificity and efficacy using clinical MRI are still unknown. An evaluation of nanoparticles with mAbs using transplanted targeted tumors without appropriate non-targeted control tumors in animals risks misevaluation, since tumor-specific microenvironmental factors, *e.g.*, the enhanced permeation and retention (EPR) effect, rich vascularization and reduced clearance [11], may induce the accumulation of nanoparticles in tumors independent of mAbs targeting. A tumor transplanted from a different genetic background may show differing microvascular formation, leading to a risk of misreading the tumor-targeting specificity. Moreover, it is essential to know how clinical MRI is applicable to these nanoparticles. Therefore, in this study we prepared appropriate controls, and conducted experiments by applying widely-used clinical (1.5-Tesla) MRI apparatus, and confirmed the results with the experimental (7.0-Tesla) MRI apparatus in order to determine accurate tumor-targeting specificity of mAb-conjugated SPIO in the present study.

To determine accurate tumor-targeting specificity *in vivo* by clinical MRI, we utilized an MRI detection system using mAb-conjugated carboxymethyl dextran magnetite (CMDM) [12], a dextran-coated superparamagnetic iron oxide (SPIO). Antibodies with internalizing functions are particularly effective for tumor targeting and therapeutics [13-14]. Recently, a new method for obtaining antibodies for tumor targeting *in vivo* was proposed, in which antibodies were screened based on their ability to bind native forms of surface antigens and to be internalized into target cells [15-16]. Because phosphatidic acid phosphatase 2a (PAP2a) is a surface antigen of pancreatic cancer cells that can be detected by antibodies screened with this new method [17-18], and antibodies with targeting ability for pancreatic cancer would be effective for early diagnosis with high sensitivity, we chose the mAbs against PAP2a obtained with this method for CMDM conjugates.

We compared positive and negative tumors derived from the same genetic background in each mouse and evaluated the tumor-targeting specificity of the mAb-CMDM conjugates using clinical 1.5-Tesla MRI. The system provided significant tumor-targeting specificity of the target tumor. Our data indicate that mAb-conjugated nanoparticles can be powerful tools for specific target tumor detection using a clinical MRI apparatus. We discuss issues regarding the specificity of the system and its future application to clinical diagnosis.

2. Materials and Methods

2.1 Cell line and mice

The a1-1 cell line, an activated H-ras gene-transduced NIH 3T3 cell line, was provided by H. Nakagama (Research Institute, National Cancer Center) [19-20]. pCacchPAP2aV1 or pCAcc was cotransfected with pBSD (blasticidin) into a1-1 cells using FuGENE6 (Roche Diagnostics, Tokyo, Japan). Stable transfectants were selected with blasticidin (0.25 $\mu\text{g/ml}$), and the clones showing the highest PAP2a expression were chosen by immunostaining (see Supplementary methods) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C with 5% CO₂. For animal experiments, 5- to 6-week-old, 20 to 22 g female Balb/c nu/nu mice (Nihon Charles River, Osaka, Japan) were injected subcutaneously with 1×10^6 a1-1 tumor cells. The animals were used in experiments on days 10 to 14 after inoculation, when the tumors were approximately 0.5 cm in diameter. All animal experiments were performed in compliance with institutional guidelines and according to the animal protocol approved by the Subcommittee on Research Animal Care at the National Center for Global Health and Medicine.

2.2 In vivo and ex vivo imaging using clinical 1.5-Tesla MRI

For MRI, the mice were injected bilaterally with a1-1 tumors that did or did not express PAP2a. For 1.5-Tesla MRI, the mice were examined after the tumors reached approximately 5 mm in diameter, and were injected with T13-CMDM-Cy5.5 or control IgG-CMDM-Cy5.5 (15 mg Fe/kg). We used a 1.5-Tesla MRI scanner with a finger coil (Magnetom Vision, Siemens, Erlangen, Germany; see Supporting Methods for additional information). The imaging protocol for the 1.5-Tesla MRI scanner consisted of coronal and transverse T_1/T_2 -weighted spin echo (SE) or turbo-SE (TSE) pulse sequences. The targeting specificity (TS) was calculated using the following formula: (positive tumor T_2 -relative intensity / negative tumor T_2 -relative intensity) $\times$ 100 – 100 (%). See Supporting Methods for additional information.

3. Results

3.1 Specific *in vivo* imaging using a fluorescent-labeled mAb targeting PAP2a

We used the mouse mAbs T13 and S11, which recognize PAP2a that is highly expressed in pancreatic cancer cells [17-18]. These antibodies were screened for tumor targeting based on their ability to bind native forms of surface antigens and to be internalized into target cells [15-16]. To prepare target cells and controls, we introduced an expression vector expressing human PAP2a or a control vector into a1-1 cells (PAP2a/a1-1 or Vec/a1-1, respectively). We obtained PAP2a or vector-stable transfectants, and their expression level was measured by immunostaining and fluorescence-activated cell sorting (FACS) analyses, which suggested that PAP2a was highly expressed in PAP2a/a1-1 cells (Fig. 1A, 1B and S1A). We used T13 for the following experiments, because T13 had a stronger affinity than S11 (Fig.S1B). To examine whether Cy5.5-labeled T13 (T13-Cy5.5) could selectively detect PAP2a/a1-1 cells *in vivo*, we transplanted PAP2a/a1-1 (positive) and Vec/a1-1 (negative) cells into nude mice bilaterally. When these PAP2a-positive and -negative tumors reached 5 mm in size, we confirmed PAP2a expression in tumors by immunohistochemical staining using T13 mAb (Fig. S1C), whereas no apparent histological change was observed in the positive and negative tumors (Fig. S1D). Subsequently, tumors were

subjected to *in vivo* fluorescence imaging analysis. To obtain accurate targeting activity, we made a simple comparison between positive and negative tumors in each mouse, and their signal intensity ratio was analyzed as targeting specificity (TS; see Materials and Methods). Selective accumulation of the fluorescent (T13-Cy5.5) signal was detected in the positive tumors from 10 min to 24 h post injection (Fig. 1C). Other independent experiments using different batches of T13-Cy5.5 provided consistent data on TS (Fig. S1E). The excised positive tumors also gave statistically significantly stronger signals than the negative tumors (Fig. 1D), indicating that T13 targeted positive tumors *in vivo*.

3.2 Stable coupling of antibodies and CMDM

CMDM is a dextran-derivative iron oxide complex approximately 40 nm in diameter (Fig. 2A), and it has an anionically charged surface (Table 1; see Supplementary Methods for CMDM preparation). To conjugate the mAb to the carboxy residues of CMDM, we examined three procedures: (i) one using sulfhydryl groups in which IgG reduced by Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and linker (a)-conjugated CMDM were incubated together; (ii) one using primary amines in which IgG and CMDM were incubated with water-soluble carbodiimide (WSC), and (iii) a third using carboxy groups of IgG in which linker (b)-conjugated CMDM and IgG were incubated with WSC (Fig. 2B and Supplementary methods). All particles including the IgG conjugation process had negative surface potentials and were comparable in diameter (50-60 nm) (Table 1). The data revealed that procedure (iii) generated the conjugate with the strongest binding affinity to PAP2a (Fig. 2C). Unfortunately, however, we noticed that the IgG was released from the CMDM-conjugate when the product of procedure (iii) was left in phosphate-buffered saline (PBS) at 4°C. The ratio of moles of IgG to moles of CMDM with procedure (iii) decreased to 0.4 from 1.7 on day 2 (Fig. S2A, Table S1). Consistently, the

Fig. 2. Schematic of the procedures and characterization of IgG-CMDM conjugates.

relative band intensities of the heavy and light chains using electrophoresis decreased (Fig. S2A (iii), left). We also observed a dissociation of the carboxymethyl dextran (CMD) from the iron oxide (Fig. S2B and S2C), suggesting that the binding between the CMD and iron oxide was not strong enough to retain the CMD, when highly hydrophilic IgG was conjugated. Therefore, we prepared CMDM with densely crosslinked dextran to ensure the stability of the dextran coat, which would minimize changes by the surrounding solutions *in vivo* [8, 21]. In this case, the ratio of moles of IgG to moles of CMDM recovered to 1.9 (Fig. S2A, Table S1), and the relative band intensities of the heavy and light chains were also partially restored (Fig. S2A (iv), right). All mAb-CMDM samples had higher ratios of moles of IgG to moles of CMDM after corsslinking (iv~v) than they did previously (i~iii) (Table 2), suggesting that mAb-CMDM conjugates became more stable due to crosslinking.

Table 1 Physicochemical characterization of IgG-CMDM conjugates.

Sample	Method	Hydrodynamic diameter (nm, mean $\pm$ SD)	Zeta potential (mV)
T13-CMDM	(i)	71 $\pm$ 34	-13.4
T13-CMDM	(ii)	56 $\pm$ 38	-13.6
T13-CMDM	(iii)	56 $\pm$ 24	-13.7
T13-CMDM	(iv)	50 $\pm$ 14	-4.1
T13-CMDM-Cy5.5	(v)	51 $\pm$ 17	-7.1
Cont. IgG-CMDM	(iv)	49 $\pm$ 15	-1.9
Cont. IgG-CMDM-Cy5.5	(v)	55 $\pm$ 22	-6.1
CMDM ^a	(iv)	47 $\pm$ 14	-7.0
CMDM (Non-crosslinked) ^b		39 $\pm$ 14	-10.0
CMDM (Crosslinked) ^c		44 $\pm$ 15	-5.6

3.3 T13-CMDM-Cy5.5 conjugates target positive tumors in vivo

Next, we examined the tumor-targeting specificity of Cy5.5-labeled T13-CMDM (T13-CMDM-Cy5.5) (see Fig. 2B (v)). Notably, T13-CMDM-(Cy5.5) and control IgG-CMDM-(Cy5.5) showed similarities in diameter, surface potential, and ratio of moles of IgG to moles of CMDM (Tables 1 and 2). We transplanted positive and negative tumors in mice; neither type of tumor showed a remarkable change in size (Fig. S4A). When T13-CMDM-Cy5.5 was injected, denser fluorescent signals were detected in positive tumors (p, arrowhead) than in negative tumors (n) except in the liver (Fig. 3A). Similar results were obtained in independent experiments (Fig. S3A). A chronological study in enucleated tumors showed that denser signals in positive tumors (p) were detected 12 to 48 h after intravenous (i.v.) administration (Fig. 3B). We evaluated TS to compare the signal intensity between positive and negative tumors in mice. The TS values were stable at over 100% after 24 h post i.v. administration (Fig. 3C). In addition, mouse whole-body imaging using experimental 7.0-Tesla MRI, and quantitative analysis using various homogenized organs by graphite furnace atomic absorption spectrometry (GF-AAS)

Table 2 The ratio of IgG mole to CMDM.

Sample	Method	The ratio of IgG mole to CMDM
T13-CMDM	(i)	0.4
T13-CMDM	(ii)	0.9
T13-CMDM	(iii)	0.4
T13-CMDM	(iv)	1.9
T13-CMDM-Cy5.5	(v)	1.8
Cont. IgG-CMDM	(iv)	1.7
Cont. IgG-CMDM-Cy5.5	(v)	1.6

and fluorescence measurements suggested that T13-CMDM-Cy5.5 had targeting specificity in positive tumors (Fig. S3B and S3C). We observed high accumulation in the liver and spleen, whereas similar accumulation was observed in control IgG-CMDM (Fig. S3B and S3C), suggesting no specificity against surface antigen. These data encouraged us to examine tumors using a clinical 1.5-Tesla MRI system.

We performed *in vivo* imaging using a 1.5-Tesla clinical MRI system following the administration of T13-CMDM-Cy5.5. To optimize the conditions for MRI measurements, we examined the signals of T₂-weighted images obtained from enucleated tumors, which were filled with water content gel to avoid an extreme decrease in MRI signal intensity due to the partial volume effect from the air environment, [22], as the TS of enucleated tumors and tumors *in vivo* were comparable (Fig. S4B and S4C). Measurement analyses of enucleated positive tumor signals using turbo spin echo (TSE) (Figs. 3D and S5A, TSE) and spin echo (SE) pulse sequences (Figs. 3D and S5B, SE) revealed that the TS of T13-CMDM-Cy5.5 was significantly higher than that of control IgG-CMDM-Cy5.5. The TS in T13-CMDM-Cy5.5 was confirmed by

fluorescence imaging (Fig. 3D, Fluorescence). *In vivo* imaging using the clinical 1.5-Tesla MRI detected stronger T₂-weighted signals in positive tumors (p) than in negative tumors (n) (Fig. 3E). By contrast, the signals from both tumors were comparable before administering the T13-CMDM-Cy5.5 (Fig. S4D). Necrosis, which causes a positive artifact in T₂-weighted signals, was not detected using the T₁-weighted signals of tumors (Fig. S4D, T₁-weighted). To obtain definite values from the MRI detection of T13-CMDM, we measured the R₂ value before and after administration of samples to obtain a ΔR_2 rate, using a 7.0-Tesla experimental MRI. Significant

Fig. 3. The T13-CMDM-Cy5.5 and T13-CMDM conjugates selectively detected positive tumors in optical and MR imaging.

accumulation of T13-CMDM was observed in positive tumors (p), whereas weak signals were detected in negative tumors (n) (Fig. 3F, see T₁- or T₂-weighted images and R₂ maps in Fig. S4E). These findings suggest that greater T₂-weighted signals were detected in positive tumors using clinical MRI because of active targeting by T13-CMDM.

4. Discussion

We visualized target tumors using clinical 1.5-Tesla MRI with mAb-conjugated nanoparticles *in vivo*. We used common cell lines with or without target molecules and side-by-side detection in the same mice to evaluate the tumor-targeting specificity of the MRI signals. To the best of our knowledge, this is the first report on the specific detection of target tumors using mAb-conjugated SPIO in well-controlled experiments using clinical MRI.

T13-CMDM exhibited significant targeting specificity (TS) using clinical 1.5-Tesla MRI, and similar results were observed for clinically well-chosen pulse sequences, fast spin echo (TSE) in this study. These data were confirmed by the measurements of Cy5.5-fluorescence imaging, 7.0-Tesla experimental MRI and homogenized tumors *in vitro*. Our data suggested that mAb-conjugated SPIO was applicable to clinical 1.5-Tesla MRI, and provided clues regarding reliable active targeting in clinical applications. A high-Tesla clinical MRI apparatus (>3.0-Tesla) has recently been introduced and provides higher resolution [23]. Nevertheless, we cannot ignore the advantages a low-magnetic field MRI apparatus, *e.g.* a wider linear correlation between MRI signals and the concentration of contrast agents, and superior homogeneity of the magnetic field compared to the high-magnetic field MRI apparatus [24]. Our data suggest that low-magnetic field clinical MRI (1.5-Tesla) is also useful and is still a good choice for MRI diagnosis.

Two tumor-targeting mechanisms have been reported. One is dependent on the tumor-specific microenvironment *e.g.*, EPR, rich vascularization (enhanced permeability) and reduced clearance (enhanced retention by immature lymphogenesis), which permit nanoparticles to accumulate within the tumor [11, 25]. The other is active targeting using peptides, ligands, or antibodies for tumor-specific molecules [26]. In this study, the EPR effect did not appear to alter T13-CMDM tumor targeting, although we cannot deny the possibility of the presence of an EPR effect via mAb-CMDM, *e.g.*, control IgG- and T13-CMDM were equally detected in negative tumors in both Cy5.5 detected-fluorescence and GF-AAS iron measurements, whereas only a weak signal was detected in CMDM without IgG. Additionally, as the T13- and control IgG-CMDM conjugates were similar in diameter, surface potential, and mAb molar ratio to that of CMDM, we conclude that our data show active targeting of mAb. Besides remarkable signal accumulation in positive tumors via T13-conjugates, nonspecific accumulation was observed in the liver and spleen. This was likely due to the characteristics of SPIO, which is sequestered in phagocytic

Kupper cells of the reticuloendothelial system [27], suggesting that tumors in the liver and spleen are contraindications of mAb-CMDM for active targeting. Conversely, we did not detect apparent signals in the pancreas, suggesting that tumors in the pancreas are available for active targeting using T13-CMDM

One essential factor for active targeting is the mAb that is used. In this study, we used the T13 mAb, which was screened based on its ability to bind native forms of surface antigens [15-16]. The T13 mAb actually exhibited targeting specificity *in vivo* as measured by both photo-imaging and MRI, suggesting that the method of screening mAb is useful for targeting the other tumor antigens *in vivo*. It is also important to elucidate how CMDM conjugates with a mAb without altering the mAb targeting ability. The conjugation of CMDM with the mAb via the carboxy groups of IgG was the best method among those that we tested, suggesting that keeping the antigen recognition site free was essential for antigen targeting. Even keeping the antigen recognition site free, *e.g.*, procedure (i) and (iii), divalent IgG exhibited more antigen binding affinity to PAP2a than the monovalent IgG in procedure (i), as previously reported [28], suggesting that total avidity was also important for antigen targeting.

Another important factor is CMDM-conjugate stability *in vivo*. Because the signals of the T13-CMDM-Cy5.5 conjugates generated greater targeting specificity after 24 h, the mAb-CMDM conjugates may accumulate gradually in the target tumors, which is consistent with a previous finding [29] and is strikingly different than conventional MRI contrast agents [30]. Therefore, mAb-CMDM conjugates have to be stable *in vivo* for more than 24 h. To stabilize the mAb-CMDM conjugates, we performed crosslinking with dextran [8, 21], and our data *in vitro* suggested that crosslinking CMD partially prevents release of mAb-CMDM from iron oxide. One reason for the dissociation of the mAb-CMDM conjugate during the incubation may be the coexistence of multiple bonding types in mAb-CMDM. The binding between antibody and CMD is represented by a covalent bond. Conversely, that between iron oxide and CMD does not involve a covalent bond [31]. This unbalanced situation may explain why CMD was released from iron oxide, when highly hydrophilic IgG was conjugated to CMD, and crosslinking of CMDM suppressed the dissociation of mAb-CMDM conjugates. Consequently, we obtained significant tumor-targeting specificity of the target tumor with T13-CMDM by both 1.5-Tesla and 7.0-Tesla MRI, although we cannot deny the possibility of additional release of mAb-CMD complex *in vivo*. We assume that the mAb-CMDM conjugate, which consists of a unified rigid bond, *e.g.*, a covalent bond, would be highly stable and would increase targeting ability *in vivo*.

A critical issue in active targeting is the development of a system that guarantees the specificity of tumor detection with high sensitivity. The TS of fluorescence detection in T13-conjugates is approximately 15-fold higher than that of MRI, suggesting that our system

worked better for targeting specific tumors with fluorescence. MRI using SPIO is inevitably susceptible to noise resulting from signals from iron or water content. We measured the total iron content of tumors using GF-AAS (Fig. S6). Since GF-AAS detected iron, including that derived from the blood (presented in data measured in the non-treated control in Fig. S6A), careful controls such as PBS or non-treated samples are necessary to estimate the Fe content derived from CMDM. The iron content in tumors and the TS of the iron in positive tumors increased significantly at 24 h, when mice were treated with T13-CMDM (Fig. S6A, S6B). Note that more than two thirds of the iron signal in positive tumors was background (Fig. S6A). These results suggest that MRI has to identify the increased signal caused by administering the mAb-conjugated SPIO from this background. This may be one of the reasons for the reduced sensitivity compared to fluorescence-dependent detection. A future trial using background-free nanoparticles coated with mAb might enable specific tumor targeting using MRI with higher sensitivity.

This study demonstrated that mAb nanodevices are useful for specifically targeting tumors by clinical 1.5-Tesla MRI and that a stable conformation of nanoparticles with targeting ability *in vivo* is important for specific tumor targeting, given that the mAb-CMDM accumulated gradually in positive tumors. The future development of background-free nanoparticles and detection systems would definitely contribute to the use of such mAb nanodevices for tumor-specific MRI diagnosis.

Acknowledgments

We thank M. Ochiai and H. Nakagama for the a1-1 cells; M. Yoneyama, T. Nakahara, S. Saito, N. Nitta, and A. Sekita for their technical support with the MRI experiments; A. Hori and P. N. Minh for performing the statistical analyses; and M. Tamura-Nakano for technical support with the immunohistochemical staining. This work was supported in part by Grants-in-Aid for Research on Advanced Medical Technology from the Ministry of Health, Labor and Welfare, Japan (Y.I.), and by funding from the JST, CREST (M.S.), and the Japan Association for the Advancement of Medical Equipment (Y.M.).

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Figure Legends

Fig. 1. mAb-specific detection of PAP2a-expressing cells and allografts in mice. **(A)** Immunostaining of a1-1 cells expressing PAP2a. After transfecting a1-1 cells with a plasmid DNA expressing PAP2a or an empty vector, clones of PAP2a/a1-1 and Vec/a1-1 cells were established. S11, a mAb against PAP2a. Top, PAP2a/a1-1; bottom Vec/a1-1. Red, PAP2a; blue, DNA. Bar = 20 μ m. **(B)** Top, fluorescence-activated cell sorting (FACS) analysis of PAP2a. T13, a mAb against PAP2a. Bottom, geometrical mean of signal intensity in PAP2a/a1-1 and Vec/a1-1 cells. ** $p = 0.004$; PAP2a/a1-1 versus Vec/a1-1. Values represent the mean $\pm$ standard deviation (SD). **(C)** *In vivo* fluorescence images of tumors after intravenous (i.v.) administration of Cy5.5-labeled T13. The color scale, Cy5.5 signal intensity. Arrows, PAP2a-positive (p) and negative (n) tumors that formed after the subcutaneous transplantation of PAP2a/a1-1 and Vec/a1-1 cells in a nude mouse. **(D)** Left, fluorescence images of enucleated positive and negative tumors. Bar = 10 mm. Right, relative Cy5.5 intensities of three independent experiments. * $p = 0.049$, positive versus negative tumors. Values represent the mean $\pm$ SD.

Fig. 2. Schematic of the procedures and characterization of IgG-CMDM conjugates. (A) Schematic of carboxymethyl dextran magnetite (CMDM). Top, CMDM; bottom, carboxymethyl dextran (CMD). (B) Procedures used for IgG-CMDM conjugation. IgG-CMDM conjugation via the (i) sulfhydryl groups, (ii) primary amines, and (iii) carboxy groups of IgG. (iv) Crosslinked CMDM version of procedure (iii). (v) Cy5.5-labeled version of procedure (iv): (a) 2-(2,5-dioxo-3,4-dihydro-pyrrol-1-yl)-ethylamine; (b) 1,6-diaminohexane; (c) epichlorohydrin (1-chloro-2,3-epoxypropane). Magenta: possible regions for the conjugation of IgG-CMDM via the primary amines of IgG; blue: possible regions for the conjugation of IgG-CMDM via the carboxy groups of IgG. (C) FACS analyses by T13-CMDM. The conjugates of T13-CMDM synthesized using procedures (i) to (iii) were used to detect PAP2a/a1-1 and Vec/a1-1 cells. Cells were incubated with antibody-conjugated CMDM ($1.0 \mu\text{g Fe}/1 \times 10^5$ cells).

Fig. 3. The T13-CMDM-Cy5.5 and T13-CMDM conjugates selectively detected positive tumors in optical and MR imaging. The T13- or control IgG-CMDM-Cy5.5 or PBS was administered i.v. to tumor-bearing mice that had been given transplants of PAP2a/a1-1 or Vec/a1-1 cells. (A) Representative optical images of whole body and extracted organs, and (B) enucleated tumors using a Fluorimager. Similar data were obtained in two other independent experiments (Fig. S3A). li: liver; s: spleen; k: kidney; pa: pancreas; n: negative tumor; p: positive tumor; b: serum. Bar = 10 mm. (C) Chronological changes in the targeting specificity (TS, see the formula in Materials and Methods) of the fluorescence after injecting T13-CMDM-Cy5.5. The values are the median and 25–75th percentiles. (D) Targeting specificity of T13-CMDM-Cy5.5 using the 1.5-Tesla MRI system. Twenty-four hours after the i.v. administration of T13-CMDM-Cy5.5 or control IgG-CMDM-Cy5.5, the tumors were enucleated and subject to fluorescence imaging analysis (left panel). MRI signals were measured using T₂-weighted images with TSE (middle panel) and SE (right panel) sequences, and then the TS was calculated. Wilcoxon rank-sum test: * $p < 0.05$; ** $p < 0.01$ ($n = 8$ – 12 for fluorescence images, $n = 8$ – 9 for TSE, $n = 5$ – 6 for SE). (E) *In vivo* T₂-weighted images detected using a 1.5-Tesla MRI with a TSE sequence, 24 h after the administration of T13-CMDM-Cy5.5. Here, 0, 3, and 6 indicate the MRI signals of phantoms that contained 0, 3, and 6 $\mu\text{g Fe/mL}$ of T13-CMDM-Cy5.5, respectively. Bar = 10 mm. (F) Quantification of T13-CMDM specificity using a 7.0-Tesla horizontal MRI scanner. T13-CMDM was administered to positive and negative tumor-bearing mice, and then R₂ maps (Fig. S4E) were acquired. The quantitative R₂ maps were subjected to the ΔR_2 rate (%): $(R_2 \text{ value } 24 \text{ h post-administration} - R_2 \text{ value pre-administration}) / R_2 \text{ value pre-administration} \times 100$; R₂: T₂ value⁻¹. Values are the median and 25–75th percentile values. * $p < 0.05$ (sign test, $n = 10$).

Table 1 Physicochemical characterization of IgG-CMDM conjugates.

Sample	Method	Hydrodynamic diameter (nm, mean ± SD)	Zeta potential (mV)
T13-CMDM	(i)	71 ± 34	-13.4
T13-CMDM	(ii)	56 ± 38	-13.6
T13-CMDM	(iii)	56 ± 24	-13.7
T13-CMDM	(iv)	50 ± 14	-4.1
T13-CMDM-Cy5.5	(v)	51 ± 17	-7.1
Cont. IgG-CMDM	(iv)	49 ± 15	-1.9
Cont. IgG-CMDM-Cy5.5	(v)	55 ± 22	-6.1
CMDM ^a	(iv)	47 ± 14	-7.0
CMDM (Non-crosslinked) ^b		39 ± 14	-10.0
CMDM (Crosslinked) ^c		44 ± 15	-5.6

Table 2 The ratio of IgG mole to CMDM.

Sample	Method	The ratio of IgG mole to CMDM
T13-CMDM	(i)	0.4
T13-CMDM	(ii)	0.9
T13-CMDM	(iii)	0.4
T13-CMDM	(iv)	1.9
T13-CMDM-Cy5.5	(v)	1.8
Cont. IgG-CMDM	(iv)	1.7
Cont. IgG-CMDM-Cy5.5	(v)	1.6

Figure 1

(A)

(B)

(C)

(D)

Figure 3