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Beta-galactosidase-responsive Synthetic Biomarker for Targeted Tumor Detection

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Tumor biomarkers are highly desirable for the screening of patients with a risk of tumor development and progression. Here, we report a beta-galactosidase (β-gal)-responsive acetaminophen (β-GR-APAP) as a synthetic plasma biomarker for targeted tumor detection. Tumor β-gal labeling via the recognition of tumor-related antigen enabled the detection of a tumor using β-GR-APAP.

Successful tumor detection relies on the identification of a valid biomarker.[1] Biological fluids such as blood or urine are easy to handle and can be used in a variety of methods for detection of endogenous biomolecules.[1b] Thus, considerable effort has been dedicated to the discovery of effective tumor biomarkers from these fluids. In particular, endogenous biomolecules such as proteins and cell-free nucleic acids are commonly used as valuable biomarkers and play essential roles in the screening of patients with a risk of tumor development.[2] However, the discovery of a reliable tumor biomarker is challenging due to the low concentration[3] or low stability of endogenous biomolecules in biological fluids.[4]

Recently, synthetic biomarkers have emerged as novel and promising tools for tumor diagnosis[5] as they bypass the fundamental challenge of conventional liquid biopsy, utilizing an *in vivo* reaction with tumor-related enzymes such as peptidases (Fig. 1a). A typical synthetic biomarker involves the integration of a peptide moiety on the surface of exogenous nanoparticle agents. Upon the reaction with peptidases in the tumor, the nanoparticle agent produces peptide fragments as reporter molecules, which are then released into the fluids and detected using various methods,[5] including mass spectrometry and enzyme-linked immunoassay for multiplex target analysis, or a paper-based test for point-of-care testing. Bhatia *et al.* reported that a synthetic biomarker for the analysis of secreted proteases such as matrix metalloproteinases enabled tumor detection at an early stage.[5e]

Despite progress in the field of synthetic biomarker development, the main candidates have thus far been limited to reactive biomolecules such as peptidases. On the other hand, many cancer cells have been shown to overexpress specific antigens without reactivity on the cell surface.[6] Cell surface antigens are related to the malignancy of cancer cells or are used as targets of antibody drugs. Therefore, these antigens are attractive candidates for synthetic biomarkers to detect targeted tumors and predict prognosis or drug efficacy.

Here, we report a novel strategy for tumor detection using a beta-galactosidase (β-gal)-responsive synthetic biomarker targeting a cell surface antigen (Fig. 1b). We focused on β-gal as this is a representative reporter enzyme[7] and β-gal conjugate can be applied to β-gal labeling of targeted tumors.[8] In addition endogenous β-gal activity in the cancer cells was used for targeted tumor detection, which also increased the positive signal.[9] Thus, a β-gal labeling strategy via the recognition of a tumor-related antigen and a β-gal-responsive synthetic biomarker might be applied to targeted tumor detection using biological fluids.

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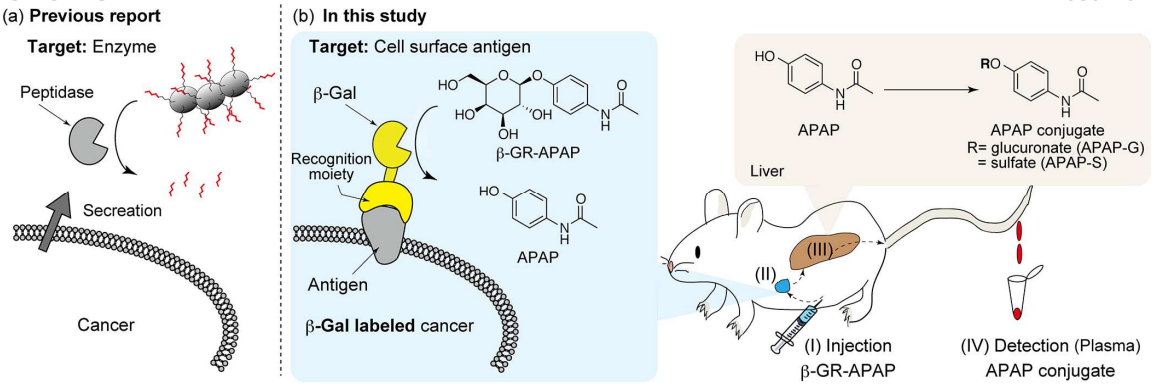
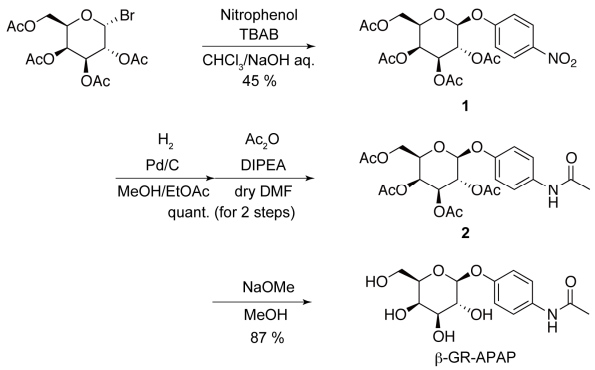


Fig. 1 Schematic illustration of tumor detection using (a) a synthetic biomarker targeting an enzyme (from ref 5) and (b) a novel synthetic biomarker targeting a cancer cell surface antigen, which is achieved using beta-galactosidase (β-gal) labeling and its reactivity. Injected β-gal-responsive acetaminophen (β-GR-APAP) releases APAP as a reporter molecule upon reaction with β-gal in the tumor tissue (I, II). APAP is then metabolized into glucuronate (APAP-G) and sulfate (APAP-S) conjugates in the liver (III). The quantification of APAP conjugates in the plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS) allows detecting the targeted tumor (IV).

We have previously proposed to design acetaminophen (APAP)-based synthetic biomarkers.[10] Based on this strategy, we designed β-gal-responsive APAP (β-GR-APAP), which released APAP upon reaction with β-gal (Fig. 1b). APAP has already been approved for administration to humans. Although the overdose of APAP causes the hepatotoxicity, basically the APAP is regarded as a safety drug.[11] Most APAP is metabolized into glucuronate (APAP-G) and sulfate (APAP-S) conjugates in the liver.[12] Therefore, the level of *in vivo* β-gal might be estimated by monitoring the level of APAP conjugates in the plasma.

Tumor detection using β-GR-APAP can be achieved as follows. After β-gal labeling of the targeted tumor tissue, β-GR-APAP is injected into mice as a synthetic biomarker (Fig. 1b, I). Injected β-GR-APAP is converted into APAP mainly in the tumor (Fig. 1b, II) and then delivered to the liver; here, APAP is metabolized into APAP-G and APAP-S (Fig. 1b, III). The quantification of APAP conjugates in the plasma by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with high sensitivity allows targeted tumor detection (Fig. 1b, IV).

To demonstrate this concept, we first synthesized β-GR-APAP from 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide in four steps (Scheme 1). Using the commercially available reagents, β-GR-APAP was synthesized with good yield.



Scheme 1 Synthesis of beta-galactosidase-responsive acetaminophen (β-GR-APAP).

As first preliminary experiments, we injected β-GR-APAP into the peritoneal cavity of mice along with purified β-gal to test the ability of detecting β-gal *in vivo* (Fig. S1). After β-GR-APAP injection, the blood was collected from mice treated with or without purified β-gal, and the plasma was analyzed by LC-MS/MS. As expected, the concentrations of the products APAP, APAP-G and APAP-S increased, and the concentration of β-GR-APAP decreased in the presence of purified β-gal. This result demonstrated that β-GR-APAP enabled the detection of *in vivo* β-gal using a plasma sample. Moreover, LC-MS/MS enabled detecting each compound with high sensitivity (Instrument detection limit = 3.4, 1.9, 0.82 and 1.8 fg; β-GR-APAP, APAP-G, APAP and APAP-S, respectively).

Our newly developed synthetic biomarker for β-gal was then applied to targeted tumor detection (Fig. 2). As a proof-of-concept, LoVo cells, a human colorectal carcinoma cell line, were used as targets. Targeted tumor cells were labeled with β-gal using avidin, which is an antigen recognition moiety that interacts with lectin on the LoVo cell surface.[13] In addition, avidin-β-gal has been used as a commercially available reagent for β-gal labeling of several tumor cells, including LoVo cells.[8,13]

The β-gal reaction was first confirmed in a cellular environment *in vitro* (Fig. 2a). After incubation with avidin-β-gal or vehicle alone, LoVo cells were washed with phosphate buffered saline. The harvested cells were incubated in a medium supplemented with β-GR-APAP to monitor the β-gal reaction. Labeled LoVo cells produced more APAP than unlabeled cells, indicating that β-GR-APAP reacted with the labeled β-gal. The ability of avidin to function as a tumor recognition moiety was confirmed by comparing the APAP production of LoVo cells labeled with avidin-β-gal and β-gal alone (Fig. S2a). The endogenous β-gal in the LoVo cells also converted β-GR-APAP into APAP, which was confirmed by using the β-gal inhibitor, 2-phenylethyl β-D-thiogalactoside, PETG (Fig. S2b).

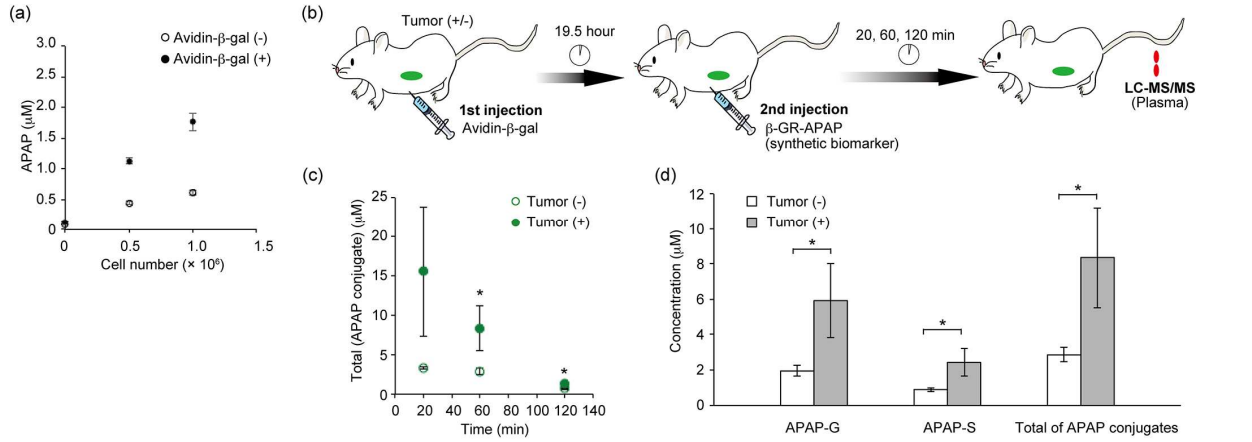


Fig. 2 (a) In vitro evaluation of β-GR-APAP to APAP conversion. The collected LoVo cells with or without β-gal labeling (0, 0.5, or 1.0 × 10^6) were incubated in Dulbecco's modified eagle medium (20 μM β-GR-APAP) for 24 h. Data are mean ± SD (n = 4 per group). (b) Tumor detection using β-GR-APAP. (c) Plasma concentrations of total APAP conjugates [glucuronate (APAP-G) and sulfate (APAP-S) conjugates] at 20, 60 and 120 min following β-GR-APAP injection. β-GR-APAP (3.6 mM, 400 μL) was injected into the peritoneal cavity at 19.5 h after avidin-β-gal injection into the peritoneal cavity (30 μg). (d) Plasma concentration of total APAP conjugates, APAP-G and APAP-S at 60 min following β-GR-APAP injection. Data are mean ± SEM (n = 6, 7). Mann-Whitney *U* test; * $P < 0.05$.

We next evaluated β-gal labeling of tumor tissues using an intraperitoneal tumor model (Fig. S3) that was established as previously reported.[14] At 19.5 h after intraperitoneal injection of avidin-β-gal, we performed X-Gal staining of the excised main organs and tumor tissues. A blue precipitate resulting from β-gal reaction in the tumor tissue was detected.

We then evaluated *in vivo* β-GR-APAP activation in the presence of LoVo cells (Fig. 2b). We first injected the mice with avidin-β-gal for β-gal labeling, and after 19.5 h, injected β-GR-APAP intraperitoneally. At 20, 60 and 120 min following β-GR-APAP injection, blood was collected from normal and tumor-implanted mice, and the plasma was analyzed by LC-MS/MS to quantify APAP conjugates.

In the present study, the concentrations of the APAP conjugates were used as specific indicators of *in vivo* β-gal conversion. The stability test of β-GR-APAP revealed that a portion of β-GR-APAP was converted to APAP in collected blood, depending on the incubation time (Fig. S4), whereas APAP-S and APAP-G were not detected, as these are only produced *in vivo*. Therefore, using the concentrations of APAP conjugates as indicators evades the problem of false positive signals produced during the preparation of plasma samples.

As shown in Fig. 2c, the concentrations of total APAP conjugates (APAP-G + APAP-S) increased in the plasma of tumor-implanted mice, and a significant difference was observed between normal and tumor-implanted mice after 60 min of incubation. The concentrations of APAP-G and APAP-S were higher in tumor-implanted than in normal mice, respectively (Fig. 2d, Fig. S5). Thus, targeted tumor detection was achieved by monitoring the plasma concentrations of APAP conjugates at 60 min following β-GR-APAP injection. To confirm the effect of tumor implantation on the production of APAP conjugates, we injected APAP into normal and tumor-implanted mice. Both groups showed similar plasma concentrations of APAP conjugates (Fig. S6) indicating that β-GR-APAP was the factor responsible for increasing the amounts of APAP conjugates, depending on the presence of β-gal in the tumor tissue.

In summary, β-GR-APAP is a novel synthetic biomarker for targeted tumor detection, as it enabled the detection of a β-gal-labeled tumor by monitoring the level of APAP conjugates in the plasma with high sensitivity. In this study, as a proof-of-concept, we used LoVo cells as targets and avidin-β-gal as the labeling agent. The proposed β-gal labeling strategy using avidin-β-gal can potentially be applied to other cancer cell lines.[8a,13]

To date, only one other synthetic biomarker utilizing a cell surface antigen has been proposed.[5e] This biomarker employed the cell surface antigen for accumulating the synthetic biomarker on the tumor tissue to facilitate analysis of the endogenous protease, while our concept is based on the direct analysis of the cell surface antigen itself via β-gal labeling using the recognition moiety.

Although the present work has only shown the potential of this strategy for detecting a relatively large tumor as a proof-of-concept, the sensitivity might be improved by using the more suitable antigen recognition moieties and the method might be applied to other types of cancer cells. Further work is currently in progress following these directions.

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Conflicts of interest

There are no conflicts to declare.

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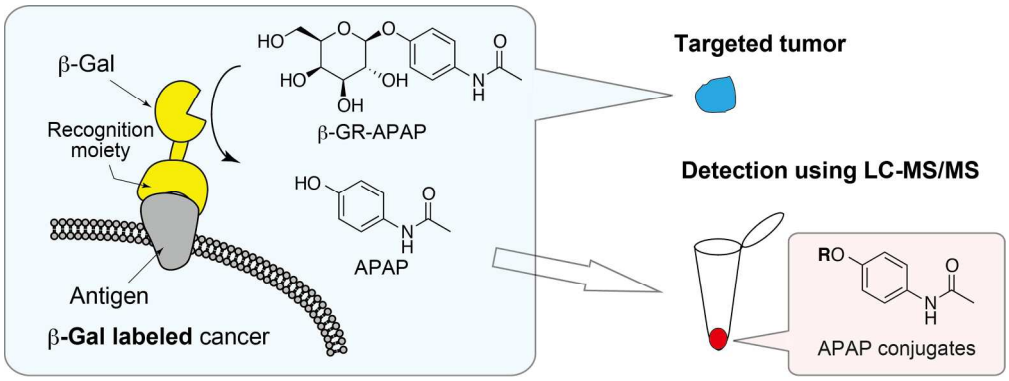

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