Molecular Photoacoustic Imaging of Follicular Thyroid Carcinoma

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Running Title: Photoacoustic Imaging of Thyroid Carcinoma

Keywords: Photoacoustic Molecular Imaging, Thyroid follicular cancer, Activatable Photoacoustic Probe, Matrix Metalloproteinase, Optical Imaging

Financial Support: This work was supported in part by National Institutes of Health Grants NCI ICMIC P50 CA114747 (SSG), CCNE U54 CA119367 (SSG), and the Canary Foundation (SSG).

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Conflict of Interest Statement: Sanjiv Sam Gambhir serves on the Advisory boards of VisualSonics and Endra.
Statement of translational relevance. Current clinical thyroid cancer diagnosis relies on ultrasound and fine needle aspiration cytology, both of which are unable to detect vascular and capsular invasion that is most often the only clear distinction between follicular thyroid carcinoma and adenoma. At present, the only method that can accurately diagnose follicular thyroid carcinoma is a biopsy after total or partial removal of the thyroid lobes. Only 20-30% of all biopsies diagnose malignancy, proving 70-80% of the surgical procedures as unnecessary. Having a non-invasive imaging method that could differentiate between benign and malignant follicular nodules would bring considerable benefit to patient management and reduce the healthcare costs by eliminating unnecessary surgeries. Photoacoustic imaging, with good depth penetration in combination with high contrast and resolution has a great potential to be clinically utilized for non-invasive, non-ionizing visualization of a superficial organ such as the thyroid.
Abstract

Purpose: To evaluate the potential of targeted photoacoustic imaging as a non-invasive method for detection of follicular thyroid carcinoma.

Experimental Design: We determined the presence and activity of two members of matrix metalloproteinase family (MMP), MMP-2 and MMP-9, suggested as biomarkers for malignant thyroid lesions, in FTC133 thyroid tumors subcutaneously implanted in nude mice. The imaging agent used to visualize tumors was MMP activatable photoacoustic probe, Alexa750-CXeeeeXPLGLAGrrrrrXK-BHQ3. Cleavage of the MMP activatable agent was imaged after intratumoral and intravenous injections in living mice optically, observing the increase in Alexa750 fluorescence, and photoacoustically, using a dual wavelength imaging method.

Results: Active forms of both MMP2 and MMP-9 enzymes were found in FTC133 tumor homogenates, with MMP-9 detected in greater amounts. The molecular imaging agent was determined to be activated by both enzymes in vitro, with MMP-9 being more efficient in this regard. Both optical and photoacoustic imaging showed significantly higher signal in tumors of mice injected with the active agent than in tumors injected with the control, non-activatable, agent.

Conclusions: With the combination of high spatial resolution and signal specificity, targeted photoacoustic imaging holds great promise as a noninvasive method for early diagnosis of follicular thyroid carcinomas.
Introduction

In the U.S. thyroid cancer has a low incidence and mortality rate compared to other cancers(1). While thyroid cancers are rare, benign thyroid nodules are very common and discriminating between the two is essential for proper patient management. Three out of four types of thyroid cancer can be accurately diagnosed using current clinical diagnostic methods that use ultrasound and fine needle aspiration cytology (FNAC) to determine cytological features indicating malignant cell transformations. However, because of its cytological similarity to benign adenoma, follicular thyroid carcinoma still presents a diagnostic challenge. Most often the only clear distinction between the follicular thyroid carcinoma and adenoma is the vascular and capsular invasion that can be detected only after partial or complete removal of the thyroid. As benign nodules are overwhelmingly prevalent, 70-80% of all surgical removals will prove to be unnecessary (2). Inclusion of a non-invasive imaging method in the clinical decision process would potentially bring considerable benefit to patient management and reduce the health care costs associated with unnecessary thyroid removals. Molecular imaging has a significant role in cancer research as a tool that enables better understanding of this disease and thus could offer insight into and improvement of various areas of cancer care (2, 3). One of the molecular imaging modalities, photoacoustic imaging, has experienced an extremely rapid growth over the recent years due to the many potential advantages it offers (4). Photoacoustic imaging, with its good tissue penetration of up to 5 cm in combination with high contrast and resolution, has a great potential to be clinically utilized for non-invasive, non-ionizing visualization of a superficial organ such as the thyroid. In this study we aim to
demonstrate the utility of biomarker-based photoacoustic imaging in the detection of follicular thyroid carcinoma in mice.

Several studies (5, 6) have suggested matrix metalloproteases as biomarkers that can differentiate between benign and malignant thyroid lesions. Using immunohistochemistry and in situ hybridization, Cho Mar et al. detected significantly higher levels of MMP-2, MMP-7 and MMP-9 in patient tissues with minimally invasive follicular carcinoma than in adenoma and adenomatous goiter tissues. Buergy et al. (6) reported increased levels of MMP-1 and MMP-9 in patient tissues with follicular thyroid carcinoma as determined by ELISA. Matrix metalloproteinases, especially MMP-2 and MMP-9, are also known to be closely associated with tumor progression and aggressiveness (7-9).

We have recently reported the synthesis and cell culture testing of a MMP activatable photoacoustic probe that showed over 13-fold higher photoacoustic signal in cells exposed to the MMP-activated probe over the ones exposed to the non-activated, un-cleaved probe (10). Activatable probes, agents that provide signal only after the activation by their target, have attracted great attention in the field of molecular imaging as they offer higher sensitivity and specificity of detection than the conventional, “always on” probes (11). Our activatable photoacoustic probe is based on dual wavelength imaging (Figure 1). Prior to reaching its target, the probe shows photoacoustic signal of the ground state complex formed between the two absorbers (10). Although non-activated probe shows signal at both wavelengths corresponding to the absorption maxima of the absorbers, the two signals are similar in intensity and the subtraction of the photoacoustic signals obtained at the two excitation
wavelengths makes the non-activated probe effectively photoacoustically silent. In the presence of a target, in this case a matrix metalloproteinase enzyme, the probe is cleaved and the observed photoacoustic signal is of the separated absorbers. The absorber that is attached to the cell penetrating peptide accumulates in nearby cells while the other absorber diffuses away, which, after the subtraction of the PA signal at the two excitation wavelengths, results in an increase in the photoacoustic signal.

The absorbers used in this study were a quencher, BHQ3, attached to the polyarginine, a cell penetrating peptide part (CPP) of the molecule and a fluorescent molecule, Alexa750, connected to the polyglutamic acid chain that does not possess cell penetrating ability. The linker between CPP and polyglutamic acid chain was the peptide sequence, PLGLAG, shown to be preferentially cleaved by MMP-2 and MMP-9 (12).
Materials and Methods

Synthesis of the activatable B-APP-A and non-cleavable, control probe. Black hole quencher, BHQ3 NHS ester was purchased from Biosearch Technology (Novato, CA), Alexa750 maleimide from Invitrogen (Carlsbad, CA) and the peptide by Protein and Nucleic acid Facility at Stanford University. To the solution of approximately 1 mg peptide (active cleavable peptide, AcCXeeeeXPLGLAGrrrrrrXK-CONH2 or non-cleavable control consisting of D-amino acids AcCXeeeeXplGlagrrrrrrXK-CONH2) in 500 μL PBS, was added a solution of 500 μg BHQ3-NHS ester dissolved in 500 μL DMF and solution of 500 μg Alexa750 maleimide in 500 μL DMF. After leaving it for 2 hours at room temperature, the reaction mixture was directly injected onto HPLC column. Analysis and purification of the probe was performed using the Dionex Summit high-performance liquid chromatography (HPLC) system (Dionex Corporation, Sunnyvale, CA). Reverse phase HPLC column Higgins Analytical (Higgins Analytical, Mountain View, CA) (C18, 4.6 mm × 250 mm) was used for the analysis of the reaction mixture. The mobile phase was 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in 90 % acetonitrile (CH3CN) in water (solvent B). The products were detected by following absorbance at 675 nm and 750 nm. Matrix assisted laser desorption/ionization mass spectrometry was performed by the Canary Center proteomics facility on AB Sciex 5800 TOF/TOF System (Foster City, CA). The probes had a retention time of 18.6 min and a molecular mass of 4 015.11 (Supplementary Figure S1) information). The concentrations of the probes for all experiments bellow were calculated using Alexa750 extinction coefficient and absorbance at 750 nm (Supplementary Figure S2).
In vitro MMP activity study. Active MMP-2 and MMP-9 were from EMD Chemicals (Gibbstown, NJ). To the probe solution (0.2 nmol/100 μL PBS) various amounts of MMP-2 and MMP-9 were added and the mixture kept at 37°C for 1 hour. The change in fluorescence was recorded using Fluoromax 4 (Horiba Scientific). The excitation wavelength was 700 nm and fluorescence was detected in the 720-800 nm window.

Photoacoustic Imaging of the probe cleavage in vitro. The active and the control probe (app. 0.6 nmol) were each dissolved in 200 μL PBS and the solution split into two vials. After adding 0.2 μg of active MMP-9 to one of the vials, all solutions were kept at 37 °C for 2 hours. Subsequent fluorescence measurements (λ_ex 720 nm) revealed increase in fluorescence only in the solution containing active probe and MMP-9 enzyme. Increase in fluorescence was not observed for the solution of the control probe with MMP-9 indicating the probe was not being cleaved by the enzyme. The solutions were injected into capillary polyethylene tubes and embedded in 1% agar. Photoacoustic imaging was performed using 680 and 750 nm wavelengths using a Visual Sonics Vevo LAZR system. To observe the probe cleavage photoacoustic images recorded at 750nm were subtracted from the images obtained at 680 nm (PA_{680nm}-PA_{750nm}).

Determination of MMP-2 and MMP-9 levels in FTC133 tumors. MMP-2 and MMP-9 assay kits were purchased from GE Healthcare. For determination of the enzyme levels in tumors, tumors were excised from mice when they reached approximately 2-3 mm diameter in size, sonicated in TRISHCl (pH 7.4) buffer, and the suspension centrifuged at 4°C and 2000xg for 10 minutes.
The absorbance of the supernatant at 280 nm was used to standardize samples by the amount of total protein.

**Optical Imaging of the probe activation in vivo.** All animal studies were performed in accordance with the Guidelines for the Care and Use of Research Animals established by the Stanford University. Optical imaging of tumor bearing mice (n=3 for cleavable B-APP-A, n=3 for the control probe) was performed using an IVIS200 system (Caliper Life Sciences, Alameda, CA). The mice were intravenously injected with 1.3 nmol or intratumorally with 0.15 nmol of the probe and imaged using ICG filter set. The exposure time for images was 1 s. Quantitative analysis was performed using the Living Image® 4.0 software.

**Photoacoustic imaging of the B-APP-A activation in vivo after intratumoral probe injection.** Mice bearing FTC 133 tumors (n=3 for B-APP-A, n=3 for the control probe) were photoacoustically imaged using a dedicated small-animal high-resolution imaging system (VevoLAZR; VisualSonics). A 256 element linear array transducer with center frequency of 21-MHz and spatial resolution of 75 µm resolution was placed to position the 10 mm focal depth in the center of the tumor. The photoacoustic gain was kept at 43 dB and dynamic range at 20 dB for all studies. The laser energy was recorded before and after each scan, and information used to normalize photoacoustic signal at different wavelengths. Ultrasound and photoacoustic images were obtained sequentially. Tumors were imaged using 680 and 750 nm laser light, prior to and 90 minutes after the intratumoral injection of 0.6 nmol probe. Images were exported as 3D volume tiff files that were stacked together using Image J. Normalization of the images by the laser energy as well as subtraction of the images recorded
at 680 and 750 nm were also done in Image J. Quantification was done using AMIDE software (13) using volumetric ellipsoid ROIs drawn based on the ultrasound images. Photoacoustic signal of the probe cleavage was obtained by subtracting images recorded at 680 and 750 nm at 90 minutes post injection and normalizing the resulting subtraction image by the pre-injection subtraction image \((\text{PA}_{680\text{nm}}-\text{PA}_{750\text{nm}})_{\text{post injection}}/ (\text{PA}_{680\text{nm}}-\text{PA}_{750\text{nm}})_{\text{pre injection}}\).

**Photoacoustic imaging of the B-APP-A activation *in vivo* after tail-vein probe injection.**

Mice bearing FTC 133 tumors (n=5 for B-APP-A, n=4 for control probe) were photoacoustically imaged using a commercial Endra photoacoustic computed tomography (PACT) system. The system uses a tunable nanosecond pulsed laser (7ns pulses, 20 Hz pulse repetition frequency, about 7mJ/pulse on the animal surface, wavelength range 680 nm to 950 nm) and 128 unfocused ultrasound transducers (with 5 MHz center frequency and 3 mm diameter) arranged in a hemi-spherical bowl filled with water. We optimized imaging protocol to suit our probe experiments using 100 views and 100 pulses/view for each wavelength (680 and 750 nm). This protocol takes 18.9 mins to acquire the data for both wavelengths. For each animal we first obtained pre-contrast data at 680 and 750 nm. After tail-vein administration of the probe (4.8 nmol), we continuously acquired data at the two wavelengths for 140 minutes. Volume rendered three dimensional (3D) photoacoustic images are reconstructed off-line using data acquired from all 128 transducers from all views using a filtered back-projection algorithm. The algorithm corrects for pulse to pulse variations in the laser intensity and small changes in the temperature that effect acoustic velocity in the water. The reconstructed 3D raw data is then analyzed using AMIDE software (13). Photoacoustic signal of the probe cleavage was obtained
by subtracting images recorded at 680 and 750 nm at a specific time post-injection and normalizing the resulting subtraction image by the pre-injection subtraction image \( (PA_{680nm} - PA_{750nm})_{\text{post injection}} / (PA_{680nm} - PA_{750nm})_{\text{pre injection}} \).
Results

The activation of the B-APP-A probe by MMP-2 and MMP-9 in vitro

In our previous work (10) we observed static quenching between the two chromophores of the activatable probe, BHQ3-APP-Alexa750 (B-APP-A). Although there is no spectral overlap between the quencher (\(\lambda_{\text{max}} = 675\) nm) and the fluorophore (\(\lambda_{\text{max}} = 750\) nm), due to static quenching, the probe in its intact state shows very low fluorescence. The absorption and photoacoustic signal of the probe are also indicative of the ground state complex formed by the dimerization of the chromophores. The cleavage of the probe can be followed optically as we observe the increase in fluorescence due to the separation of Alexa 750 from the quencher. The probe was more efficiently cleaved by MMP-9 than by MMP-2 (Figure 2A). We observed close to a 9-fold increase in fluorescence intensity after incubation of the probe with 0.1 \(\mu\)g of MMP-9 for one hour at 37 °C. The same amount of probe required 20 times higher quantity of MMP-2 to achieve the same level of increase in fluorescence intensity (Figure 2B). Besides MMP-2 and 9, PLGLAG sequence is known to be cleaved by other members of the MMP family(12). MMP-7 and MMP-13 that are also associated with thyroid cancer (6, 14) have shown good cleavage profiles although they were both less efficient than MMP-9 (Supplementary Figure S3).

To show that the activation of the probe in vitro can be followed photoacoustically we created an agar phantom with embedded tubes carrying solutions of the active and the control probes before and after the cleavage with MMP-9 (Figure 2C,E). Increase in subtraction photoacoustic signal (\(\text{PA}_{680\text{nm}} - \text{PA}_{750\text{nm}}\)) was observed only for the active B-APP-A probe exposed to MMP-9.
This result shows that cleavage only, without removal of Alexa 750, leads to the increase in subtraction signal (Figure 2E).

**MMP-2 and MMP-9 levels in FTC 133 tumor homogenates**

We chose to study the activation of the probe in follicular thyroid carcinoma using a FTC133 tumor model. Levels of MMP-2 and MMP-9 in tumor homogenates were determined using an immunocapture assay (15).

Active forms of both MMP-2 and MMP-9 were observed in tumor homogenates (Figure 3) with MMP-9 detected in greater amount. Determination of the total enzyme level that includes both the proactive and the active forms suggested the predominance of the active form of MMP-9 in follicular tumors (Supplementary Figure S4). Although cleaved by both enzymes, considering higher level of the active form of MMP-9 in tumor homogenates and the better cleavage efficiency (Figure 2) we expect the probe to be primarily cleaved by the MMP-9 in FTC133 tumors.

**Optical imaging of the probe activation in vivo**

Because the probe cleavage produces both photoacoustic and fluorescent signal (Figure 2), we were able to follow activation of the probe using optical imaging (Figure 4). It needs to be stressed that the probe was not designed for fluorescent imaging as the chromophore that accumulates in cells is a quencher that is not detectable via optical imaging. However, the cleavage of the probe can still be followed optically by detecting the fluorescence of the fluorophore, Alexa 750 as it detaches from the quencher and clears away. Mice bearing
Follicular thyroid tumors were injected with the active probe B-APP-A and the control, a non-cleavable probe having the same amino acid sequence but composed of D-amino acids. Fluorescence signal at the tumor site of mice injected with the control probe was significantly lower than the fluorescence signal in mice injected with the active probe (Figure 4 A,B). High fluorescent signal was observed in the kidneys signifying probable clearance route (Supplementary Figure S5). Similar results were observed in the study that investigated proteolytic activation of a related cleavable peptide by optical imaging(16). The clearance through and possible accumulation in the kidneys could lead to systemic toxicity hypothesized to result from the non-specific cleavage of the probe followed by mast cell degranulation (12). Systemic toxicity could be minimized by reducing the non-specific cleavage through containment of the probe proteolysis within tumor by means of intratumoral injections, or by improving pharmacokinetic behavior through attachment of macromolecules, such as polyethylene glycol, to the probe to minimize renal clearance(17).

We tested the intratumoral activation of the probe by injecting 0.15 nmol of the probe directly into FTC 133 tumors and following the fluorescence increase over time (Figure 4 C,D). Fluorescence signal obtained from the tumors injected with the cleavable probe was higher than the signal detected in the tumors injected with the control probe at all time points becoming significantly different at 1.5 hours post injection.

**Photoacoustic imaging of the probe activation in living mice**

Tumors were photoacoustically imaged using 680 and 750 nm light, prior and 90 minutes after injecting 0.6 nmol probe directly into the tumor. The pre-injection images served as
normalization factors for our post injection images. Signal acquired at 750 nm was subtracted from the signal recorded at 680 nm to obtain photoacoustic signal corresponding to the cleavage of the probe. Tumors injected with the active probe showed significantly higher normalized subtraction PA signal than the tumors injected with the control probe (Figure 5 A,B). It needs to be pointed out here that the injection of either of the probes leads to increase in photoacoustic signal at individual wavelength (Supplementary Figure S6). The difference between the two probes caused by the cleavage became apparent only upon subtraction of the images acquired at 680 and 750 nm.

Thyroid tumors were also photoacoustically imaged continuously for 140 minutes after the tail-vein injection of 4.8 nmol of the probes (Figure 6). At early time points, we did not observe any difference between active and control probe. Over time, the signal for active probe increased steadily, becoming significantly different than the signal for the control probe at 100 minutes post injection (Figure 6B).

Discussion

The targets that we investigated were extensively studied members of the matrix metalloproteinase family, MMP-2 and MMP-9, which have been suggested as markers able to distinguish between follicular thyroid carcinoma and adenoma (5, 6). Using quantitative immunocapture assay, we have determined MMP-9 to be the dominant form in FTC133 tumors.

In a recent study Razansky et. al. (18) employed multispectral optoacoustic tomography to image atherosclerotic plaques ex vivo using commercially available MMP activated probe
MMPSense™ 680 intended for optical imaging. In the current study we used photoacoustic probe based on dual wavelength imaging activated by the MMP cleavage of the peptide linker. Based on the efficiency of the proteolysis and the high quantities of the active form present in FTC 133 tumors we expect the probe to be preferentially activated by MMP-9 in living subjects.

Two recent studies (19, 20) have reported possible non-specific cleavage for probes of similar design that would lead to diminished target specificity of our probe. One study suggested the breakdown of the BHQ3 in blood plasma, rather than the MMP cleavage of the probe, to be a process that leads to the fluorescence emission (20). Optical imaging of tumor bearing mice injected with the active and the control probe gave insight into the extent and rate of BHQ3 breakdown in our probe. Similarly to the MMP cleavage, the breakdown of the BHQ3 would result in the increase in fluorescence, and if it were a preferential activation process the same level of fluorescence should be observed for both the active and the control probe. Because we observed a 2-fold higher fluorescence signal in mice injected with the active probe, we reason that at this time point the breakdown of BHQ3 was not contributing significantly to the increase in fluorescence signal. The other study by van Duijnhoven et al. pointed to a possibility of a non-specific tumor accumulation of the probe caused by the tumor-independent activation most likely occurring in the vasculature (19). Although this study reported results only at one time point, 24 hours post-injection, when the non-specific cleavage contribution may be more significant than at earlier time points at which we image, to minimize any possible non-specific cleavage we investigated direct intratumoral injections. The delivery of the imaging probe should not present a problem as thyroid nodules are easily accessed using well established clinical method, ultrasound guided fine needle aspiration. Intranodular injections would localize
activation of the probe and would thus provide the information on the MMPs activity only in
the nodule. In addition, the study by Lo et al. suggested that intratumoral injection would
minimize any BHQ3 breakdown as they reported very little non-specific cleavage 24 h after
intratumoral injection of the BHQ3 labeled peptide (21).

Optical imaging of mice intratumorally injected with the active and the control probe suggested
90 minutes post-injection to be the optimal time for imaging the difference in cleavage
between the two probes. The signal coming from the tumors injected with the cleavable probe
was higher at all time points than the signal from the tumors injected with the non-cleavable
probe. While the signal for the B-APP-A probe increased over 90 minutes and then slowly
decreased at 6 hours p.i., the control probe had a stable fluorescent signal decreasing at a
slower rate. The increase in fluorescence observed at 90 minutes for B-APP-A probe can be
attributed to the cleavage of the probe and release of Alexa 750 from the ground complex.
Being smaller than the intact probe, the polyanionic part carrying Alexa 750 clears more
efficiently from the tumor site causing a drop in the fluorescence intensity. The fluorescent
signal of the control probe shows slower decay as a result of the intact probe’s delayed
clearance. The fluorescent signal in the bladder of B-APP-A injected mice was considerably
higher than the signal in control probe injected mice suggesting differential clearance between
the two probes (Supplementary Figure S7).

Although the similarity of the difference in fluorescence increase between the active and the
control agent after intravenous and intratumoral injection (2.06 vs. 2.09 fold, Figure 4C and 4D)
suggested there was no tumor nonspecific activation at the time point of imaging, we
investigated photoacoustic imaging of tumors after intranodular injection because that strategy is clinically feasible and can provide reduction in potential systemic toxicity. Photoacoustic imaging after intratumoral injection showed clear distinction between tumors injected with the active and the control probe.

Continuous photoacoustic imaging after tail vein injection clearly differentiated between the two probes. At early time points after the injection, the active and the control agent showed similar photoacoustic signal as there was no appreciable accumulation of the quencher in tumors. Over time, with more active probe being cleaved by the MMPs, higher levels of the quencher accumulated at the cleavage site leading to an increase in the photoacoustic signal. Because the control probe cannot be cleaved, it cleared away from the tumor site without significant accumulation of the quencher resulting in no increase in photoacoustic signal over time. Significant difference in signal between the active and the control probe was observed 100 minutes post injection, which represents the time suitable to image MMP activity. Although for clinical translation the optimal imaging time would have to be re-determined, the imaging time point of 100 minutes post injection in mice indicates that in humans the most favorable imaging time would be within clinically relevant time of few hours post injection of the probe. The difference between the active and the control probe was more pronounced after the intratumoral injection than after the intravenous one, most likely because of the differential pharmacokinetic behavior. Because it’s circulating in the blood, only a portion of the intravenously injected active probe gets cleaved in the tumors. On the other hand, intratumoral injections confine the probe to the tumor and slow down its clearance allowing more time for the cleavage and accumulation. The images in Figure 5 and 6 effectively illustrate the superior
resolution achieved with photoacoustic imaging. Although optical imaging provides information about MMP activation as well (Figure 4) the details about the exact location of the signal are lacking.

There are several issues that need to be addressed in the future studies. Both fluorescent and photoacoustic signal in tumors intratumorally injected with the imaging probes varied greatly between mice. Non uniform intratumoral injection was likely a major reason for the observed variation. A more efficient intratumoral delivery method will likely be needed to minimize this variation. In addition, although we investigated the cleavage of the probe in non-thyroid tumors with high and low MMP levels (Supplementary Figure S8), because of the lack of good animal models for thyroid adenomas we did not evaluate the probe in benign tumors. To show the ability of the probe to differentiate between carcinomas and adenomas, in future studies we plan to expose the probe to normal and cancer tissue extracts as sources with differential levels of MMPs. The planned study will also be used to correlate the level of MMPs to the photoacoustic signal, a link that needs to be determined for a complete quantitative evaluation of the utility of the biomarker-based molecular imaging strategy.

In conclusion, we report here the photoacoustic molecular imaging of the follicular thyroid carcinoma using an activatable photoacoustic probe. A large variety of photoacoustic agents, mainly nanoparticles, have been developed and used in various imaging applications (22-25). To the best of our knowledge this the first report of the use of a small molecule-based activatable photoacoustic probes in living subjects. The agent was efficiently cleaved by the MMPs and the activation as well as the subsequent accumulation successfully photoacoustically imaged.
Offering a combination of the high spatial resolution and signal specificity, targeted photoacoustic molecular imaging holds great promise as a noninvasive method for diagnosing follicular thyroid carcinomas.

References

Figure Legends

**Figure 1. Scheme illustrating the probe design and mechanism of action.** Non-activated probe produces a photoacoustic signal at two wavelengths ($\lambda_{A1}, \lambda_{A2}$) corresponding to the absorption maxima of the two chromophores A1 (BHQ3) and A2 (Alexa750). After cleavage, the cell penetrating peptide (CPP) portion of the probe, carrying one of the chromophores, accumulates in cells and results in a photoacoustic signal at only one of the two wavelengths $\lambda_{A1}$. In the peptide, CXeeeeXPLGLAGrrrrrXK, small letters denote D amino acids, X is a 6-aminohexanoyl acid.

**Figure 2. The activation of the B-APP-A probe by MMP-2 and MMP-9 in vitro.** The probe was more efficiently cleaved by MMP-9 (0.1 µg) than by MMP-2 (0.1 µg) enzyme (A). Complete cleavage of the probe as judged by the largest increase in fluorescence intensity was achieved using 2 µg MMP-2 (B) and 0.1 µg MMP-9 (C). The cleavage of the active and the control probes with MMP-9 was tested by fluorescence (D) and imaged photoacoustically (E). Polyethylene tubes containing approximately 0.6 nmol of the active and the control probes with and without MMP-9 were embedded in agar phantom (brightfield image shown on the left in E) and imaged using light of 680 and 750 nm wavelength. The subtraction image (680-750 nm) showed PA signal only in the capillary tubes carrying active probe, B-APP-A and MMP-9.

**Figure 3. MMP-2 and MMP-9 levels in FTC133 tumor homogenates.** Tumor homogenates had higher level of MMP-9 present. MMP-9 existed in predominately active form (Supporting Information). The error bars represent standard deviation (n=3).
Figure 4. Optical imaging of the probe activation in FTC 133 tumors. Mice bearing FTC 133 tumors in the hind legs were intravenously injected with 1.3 nmol of the B-APP-A or the control probe, and optically imaged using ICG filter set (710-760 nm excitation and 810-875 nm emission) with 1s exposure time. (A) One and a half hours post injection there was a considerably higher fluorescent signal in the tumors of mice injected with the cleavable B-APP-A probe than in the mice injected with the control probe. (B) Quantitative analysis revealed 2 fold higher fluorescent signal in the tumors of mice injected with the B-APP-A probe than of the mice injected with the control probe. The error bars represent standard deviation. * p< 0.05, n=3. Intratumoral injection of 0.15 nmol of the B-APP-A (C) and the control probe (D) was optically imaged using ICG filter set (710-760 nm excitation and 810-875 nm emission) with 1s exposure time. (E) One and a half hours post injection there was a significantly higher fluorescent signal in the tumors of mice injected with the cleavable B-APP-A probe than in tumor of mice injected with the control probe. Error bars represent standard deviation (n=3)* p< 0.05. The small images in (C) and (D) are fluorescent images of the B-APP-A control probe prior to intratumoral injection and show the same level of fluorescence intensity for both probes.

Figure 5. Photoacoustic imaging of the probe activation in tumor after intratumoral injection of the probe. Mice bearing FTC 133 tumors in the hind legs were photoacoustically imaged using 680 and 750 nm light before and after the intratumoral injection of 0.6 nmol B-APP-A or the control probe (A). Grey outline represents ultrasound image of the tumor, while the rainbow scale corresponds to the detected photoacoustic signal. At 90 minutes post injection the subtraction of the images at the two wavelengths led to a clear increase in photoacoustic
signal for the active probe. The increase in signal was not observed after the injection of the control probe. Quantification of the photoacoustic signal showed significantly higher signal after injection of the cleavable probe than after injection of the control probe. Subtraction photoacoustic signal was normalized by the pre-injection subtraction photoacoustic signal \([(PA_{680\text{nm}}-PA_{750\text{nm}})_{90 \text{ min post injection}}] / [(PA_{680\text{nm}}-PA_{750\text{nm}})_{\text{pre injection}}]\). The error bars represent standard deviation (n=3, * p< 0.05). Scale bar is 0.25 cm.

**Figure 6. Photoacoustic imaging of the probe activation in tumor after tail-vein injection of the probe.** Mice bearing FTC 133 tumors in the hind legs were photoacoustically imaged using 680 and 750 nm light before and after the injection of 4.8 nmol of the B-APP-A and the control probe (A). The subtraction photoacoustic signal at 140 minutes p.i. was approximately 1.7 fold higher than the pre injection signal for the active probe. The subtraction signal for the control probe did not change over time. At early time points the difference in subtraction signal was not significantly different for the two probes. Over time the signal for activatable probe steadily increased, becoming significantly different at 100 minutes (B). Subtraction photoacoustic signal was normalized by the pre-injection subtraction photoacoustic signal \([(PA_{680\text{nm}}-PA_{750\text{nm}})_{\text{post injection}}] / [(PA_{680\text{nm}}-PA_{750\text{nm}})_{\text{pre injection}}]\). The error bars represent standard error (n=5 for B-APP-A, n=4 for control probe, * p< 0.05).
Figure 1

PA signal

\[ \lambda_{A1} \quad \lambda_{A2} \]

MMP-2

\[ \lambda_{A1} \quad \lambda_{A2} \]

Cells

\[ \lambda_{A1} \]

\[ = \text{Alexa750-CXeeeeXPLGLA} \text{GrrrrrXK-BHQ3} \]
Figure 2
Figure 3

Graph showing enzyme level (ng/mL) for MMP-2 and MMP-9.
Figure 6

(A) Pre injection and post injection images of B-APP-A and control groups. The images show the normalized PA signal at different time points.

(B) Graph showing the normalized PA signal over time for B-APP-A and control groups. The signal is normalized to 0.5 at the start (min) and reaches a maximum at 140 min. The B-APP-A group shows a statistically significant increase in signal compared to the control group (* significance).
Clinical Cancer Research

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Clin Cancer Res Published OnlineFirst January 24, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-3061

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