

Relationship between Apoptosis Imaging and Radioiodine Therapy in Tumor Cells with Different Sodium Iodide Symporter Gene Expression

Kyung Oh Jung, Hyewon Youn, Young-Hwa Kim, Seunghoo Kim, Juri Na, Yong-il Kim, Jin Woo Park, Keon Wook Kang, Dong Soo Lee, and June-Key Chung

Abstract

The therapeutic efficacy of radioiodine (^{131}I) therapy has been reported to be variable among cancer patients and even between metastatic regions in the same patients. Because the expression level of sodium iodide symporter (NIS) cannot reflect the efficacy of therapy, other strategies are required to predict the precise therapeutic effect of ^{131}I therapy. In this research, we investigated the correlation between iodine (I) uptake, apoptosis imaging, and therapeutic efficacy. Two HT29 cell lines, cytomegalovirus (CMV)-NIS (or NIS+++) and TERT-NIS (or NIS+), were established by retroviral transfection. I uptake was estimated by I-uptake assay and gamma camera imaging. Apoptosis was evaluated by confocal microscopy and a Maestro fluorescence imaging system (CRi Inc., Woburn, MA) using ApoFlamma (BioACTs, Seoul, Korea), a fluorescent dye-conjugated apoptosis-targeting peptide 1 (ApoPep-1). Therapeutic efficacy was determined by tumor size. The CMV-NIS showed higher I uptake and ApoFlamma signals than TERT-NIS. In xenograft models, CMV-NIS also showed high 99m technetium signals and ApoFlamma signals. Tumor reduction had a stronger correlation with apoptosis imaging signals than with gamma camera imaging signals, which reflect I uptake. Higher NIS-expressing tumors showed increased apoptosis and I uptake, resulting in a significant tumor reduction. Moreover, tumor reduction showed a strong correlation with ApoFlamma imaging compared to I-uptake imaging.

RADIOIODINE (^{131}I) has been effectively used in patients with differentiated thyroid carcinomas for more than 70 years.¹ The molecular mechanism behind iodine (I) uptake in cancer cells was mediated by the membrane protein sodium iodide symporter (NIS). As a consequence, the relationship between cellular NIS expression and the therapeutic effect of ^{131}I has been studied to improve the efficacy of ^{131}I therapy.^{2,3}

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The therapeutic effect of ^{131}I on residual/metastatic lesions of differentiated thyroid cancer patients is variable among patients.⁴ For example, one-third of pulmonary metastases were cured in the Seoul National University Hospital, but 8% of the tumors showed aggressive characteristics.⁵ NIS expression seems to provide important information on the therapeutic effect of ^{131}I . However, NIS expression cannot currently successfully reflect the therapeutic efficacy of ^{131}I for various reasons, including insufficient thyroid-stimulating hormone stimulation or the failure of a low-I diet.^{6–8} Consequently, other strategies are required to predict the precise therapeutic effect of ^{131}I rather than relying on NIS expression.

As apoptosis is an essential process in cancer therapy,⁹ apoptosis imaging provides important prognostic information.¹⁰ Although annexin V has been widely used for apoptosis imaging in clinical circumstances, annexin V, which targets phosphatidylserine in the inner leaflet of cell membrane, has many disadvantages in terms of a lack of specificity for apoptotic cells to nonapoptotic cells, slow removal from the body, and a low signal to background ratio for in vivo practice.^{11,12}

ApoFlamma (BioActs, Seoul, Korea) is a new apoptosis-targeting peptide 1, named ApoPep-1, with six amino acids (CQRPQR) conjugated to a fluorescent dye (Figure S1, online version only). ApoPep-1 targets apoptotic cells by binding to histone H1 exposed on the surface of apoptotic cells but does not compete with annexin V (Figure S2, online version only).¹³ ApoFlamma was stable in serum and did not bind to either white or red blood cells.^{14,15} Unlike bulky annexin V, ApoFlamma shows efficient tissue penetration, rapid clearance from blood circulation, and low immunogenicity, making it a useful probe for in vitro and in vivo imaging.^{16,17}

In the present study, a human colorectal adenocarcinoma cell line, transfected with two different retroviral NIS vectors that show different levels of NIS expression, underwent ¹³¹I gene therapy. The correlations among I uptake, apoptosis and tumor size reduction were analyzed to predict the therapeutic efficacy of ¹³¹I treatment in tumors expressing different levels of NIS.

Materials and Methods

Establishment of Cell Lines Expressing Different Levels of NIS

The human colorectal adenocarcinoma cell line (HT29) was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea) and infected with retrovirus having the TERT promoter (weak) or the CMV promoter (strong)¹⁸ for differing degrees of NIS expression according to the manufacturer's instructions (BD Biosciences Clontech, Palo Alto, CA) (Figure 1A). Stable cells were isolated from puromycin selection (1.5 g/mL) and confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot (Figure 1B). The HT29 cell lines with higher NIS expression were labeled as CMV-NIS (or NIS+++) cells, whereas cell lines with lower NIS expression were labeled as TERT-NIS (or NIS+) cells. PCR primers for hNIS were ACTTTGCAGTACATTGTAGCC (sense), ACAGTGACTGCAGCCATAG (antisense); β -actin TCTACAATGAGCTGCGTGTG (sense), TAGATGGGCACAGTGTGGGT (antisense). Antibodies for hNIS (Abcam, Cambridge, MA) or β -actin (Cell Signaling, Danvers, MA) and antimouse antibody (Invitrogen-Molecular Probes, Eugene, OR) were used for Western blot.

In Vitro ¹²⁵I Uptake Assay

To assess human NIS activity, TERT-NIS and CMV-NIS cells were seeded in 24-well plates. After 24 hours'

incubation, cells were washed with phosphate-buffered saline (PBS) and replaced with 500 μ L of Hank's Balanced Salt Solution (HBSS) buffer containing 0.5% bovine serum albumin and 10 mM of 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid-NaOH (pH 7.4). Following this, 500 μ L of 10 mM NaI and carrier-free Na¹²⁵I (at a specific activity of 3.7 kBq [0.1 mCi]) were added to the cells and incubated for 30 minutes at 37°C. Cells were then quickly washed twice with ice-cold iodide-free HBSS, and 0.2% sodium dodecyl sulfate (SDS) was added. ¹³¹I activity was measured by a gamma counter (Cobra II, Canberra Packard, Meriden, CT) and normalized to protein content.

In Vitro Confocal Microscopy Imaging

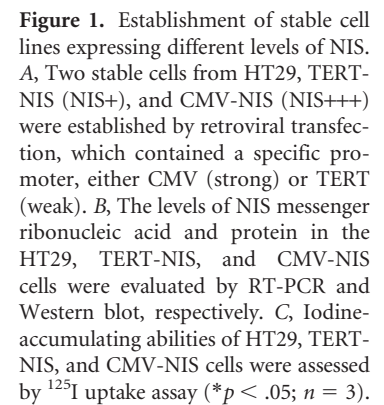
Apoptosis was imaged by ApoFlamma fluorescence with confocal microscopy. Tumor cells were seeded in 24-well plates. Following 24 hours' incubation, cells were washed with HBSS once and incubated with 500 μ L of HBSS containing 37 MBq (0.1 mCi) of ¹³¹I and 20 μ M NaI for 7 hours at 37°C to induce apoptosis. Cells were washed twice with HBSS and once with PBS. Cells were then incubated with 1 mL of ApoFlamma (648 μ g/mL) for 1 hour. Cells were fixed with 3.7% formaldehyde, and the ApoFlamma fluorescent signals (FPR-648) were analyzed by the Zeiss LSM510 META confocal imaging system (Carl Zeiss, Thornwood, CA). Further analysis was performed by *MetaMorph* software (Molecular Probes, Portland, OR) and the immunofluorescent cell population analysis system (TissueFAXS, TissueGnostics, Vienna, Austria).

Tumor Xenograft in Nude Mice

All in vivo procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University in accordance with the guidelines for the care and use of laboratory animals. Tumor cells were subcutaneously transplanted into 5-week-old adult male BALB/c nude mice weighing 20 g on average. The two different NIS-expressing tumor cells were transplanted in both the right (CMV-NIS) and left (TERT-NIS) thighs of each mouse. When tumor xenograft reached 10 mm in diameter, mice were treated with ¹³¹I (1.6 mCi) to induce apoptosis.

Tumor Size Measurement

Tumor sizes were measured with calipers weekly and calculated according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria. Tumor growth was



¹²⁵I-uptake assay revealed that the I-accumulating ability of the CMV-NIS cells was significantly higher than that of the TERT-NIS tumor cells (Figure 1C). In the TERT-NIS cells, there was no significant difference in cellular apoptosis between the ¹³¹I-nontreated and treated cells as determined by ApoFlamma imaging (Figure 2A). In contrast, ¹³¹I-treated cells in CMV-NIS cells (Figure 2B) had more intense ApoFlamma fluorescent signals (approximately 2.5-fold, quantified by *MetaMorph*) compared to untreated cells. These results indicate that apoptosis occurred more vigorously in tumor cells with high levels of NIS expression (CMV-NIS) compared to those with low levels of NIS expression (TERT-NIS). To analyze cellular apoptosis in tumor cells resulting from ¹³¹I treatment, fluorescent images of ApoFlamma were scanned and analyzed by TissueFAXS (Figure 2C). Approximately 55% of ApoFlamma-positive cells were observed in cells with CMV-NIS after 24 hours of ¹³¹I treatment, whereas only 1% of ApoFlamma-positive cells were observed in cells with TERT-NIS. This result indicated that apoptosis

measured by ApoFlamma imaging is positively related to NIS expression and I uptake. Interestingly, the cellular level of apoptosis measured by ApoFlamma seems to be quite heterogeneous.

In Vivo Gamma Camera Imaging and ApoFlamma Imaging of Tumors with Different NIS Expression

To evaluate the ability of ^{131}I uptake from tumors with different NIS expression, $^{99\text{m}}\text{Tc}$ uptake was estimated by gamma camera imaging as an indicator of NIS activity (Figure 3A). From ROI of the two tumors drawn on gamma camera images, significantly more (1.9-fold) gamma radiation signal was observed in the CMV-NIS tumors compared to the TERT-NIS tumors. For measuring apoptosis in vivo, ApoFlamma was injected intravenously 3 days after ^{131}I treatment. Two hours later (Figure 3B), the tumors with CMV-NIS showed significantly higher fluorescent signals (approximately 1.4 times higher) than tumors with TERT-NIS.

Correlation between Estimated NIS Activity and Apoptosis after ^{131}I Treatment In Vitro and In Vivo

From ^{131}I uptake measurement and ApoFlamma imaging after ^{131}I treatment, CMV-NIS showed more ^{131}I uptake and apoptotic cells both in vitro and in vivo (Figure 3, C and D). The cell line with TERT-NIS showed less differences in I uptake and apoptosis imaging. However, the cell line with CMV-NIS expression also showed more ^{131}I uptake and apoptosis than TERT-NIS, but the increase in the fold of apoptosis in CMV-NIS after ^{131}I treatment was much less than the increase in the fold of ^{131}I uptake when compared to TERT-NIS.

Therapeutic Effect of ^{131}I in Tumor Models

Three days after ^{131}I treatment, there was no distinguishable difference in tumor size between the TERT-NIS tumor and the CMV-NIS tumor (Figure 4A). However, the ApoFlamma fluorescent signals in the CMV-NIS tumors

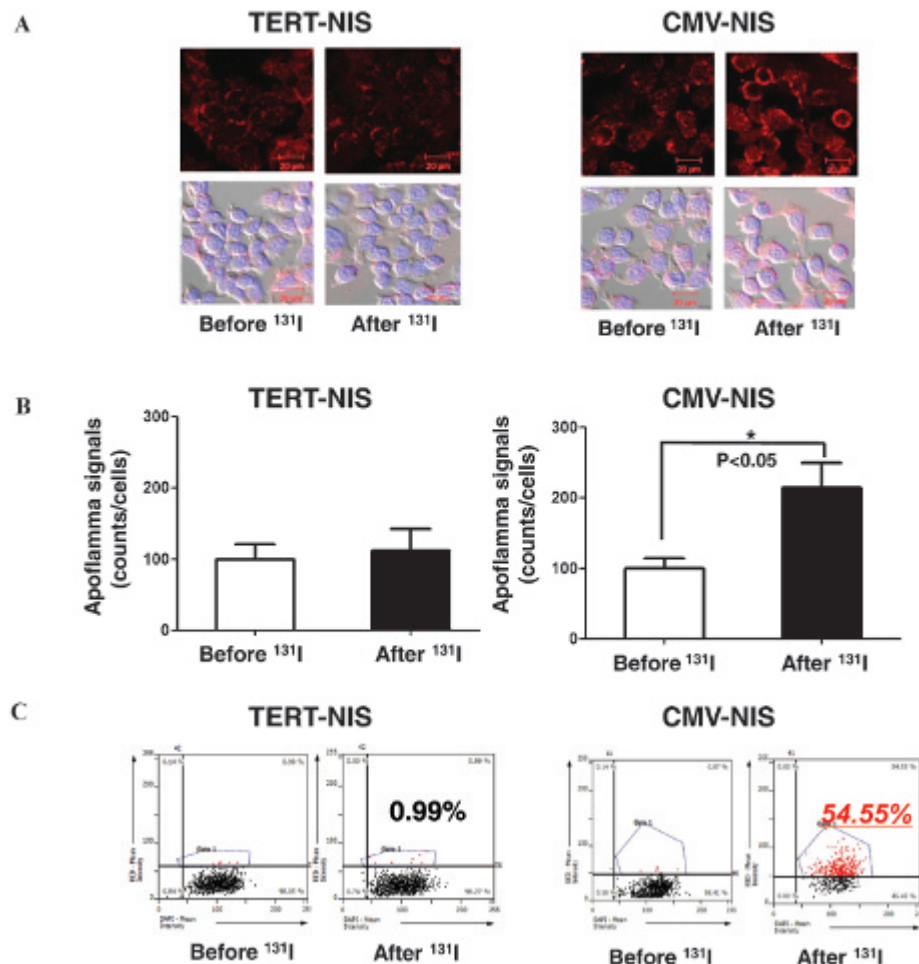


Figure 2. In vitro apoptosis imaging by ApoFlamma in tumor cells expressing different levels of NIS. **A**, Apoptosis induction by ^{131}I was imaged with ApoFlamma using confocal microscopy. ApoFlamma was applied to tumor cells at 24 hours after ^{131}I treatment (0.1 mCi). **B**, ApoFlamma fluorescent signals were further analyzed and quantified by MetaMorph software ($*p < .05$; $n = 3$). **C**, For population analysis of cellular apoptosis in each NIS-expressing cell, ApoFlamma images were scanned and analyzed by the immunofluorescence cell population analysis system (TissueFAXS).

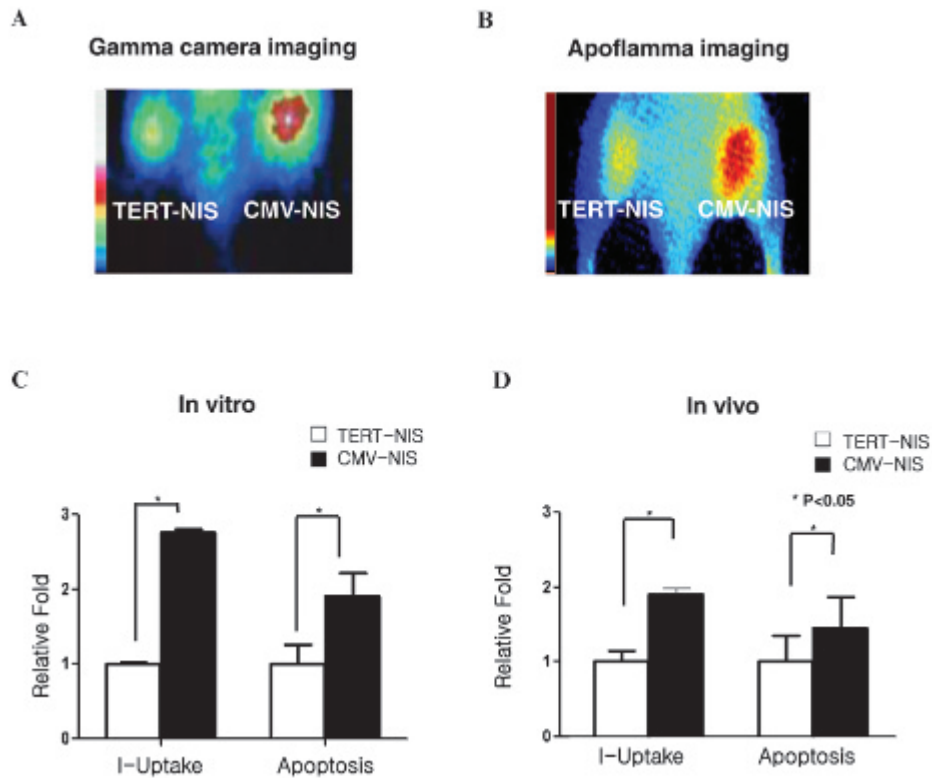


Figure 3. The correlation between iodine uptake and ApoFlamma fluorescence imaging in tumors expressing different levels of NIS (* $p < .05$; $n = 3$). *A*, In vivo gamma camera imaging of tumor-bearing mice was used as an indicator of NIS activity and acquired before ^{131}I treatment. *B*, In vivo fluorescent imaging obtained by the Maestro system. ApoFlamma was intravenously injected into mice 3 days after ^{131}I treatment (1.6 mCi). *C*, Iodine uptake and apoptosis were compared in vitro. *D*, Iodine uptake and apoptosis were compared in vivo.

were significantly more intense than the TERT-NIS tumors, indicating that more apoptosis occurred in the higher NIS-expressing tumors (Figure 4B). Three weeks after ^{131}I treatment, the size of tumors with CMV-NIS significantly decreased compared to the size of tumors with TERT-NIS, indicating that the therapeutic effects of ^{131}I treatment were more successful in high levels of NIS-expressing tumors than in low levels of NIS-expressing tumors (see Figure 4, A and B). As ^{131}I uptake and apoptosis were positively correlated with therapeutic efficacy, gamma camera imaging and ApoFlamma imaging were compared to the size of the tumor at 3 weeks after therapy. Both radioisotope uptake and ApoFlamma signals in different levels of NIS-expressing tumors were significantly correlated to tumor size reduction 3 weeks after ^{131}I treatment (Figure 4, C and D).

Discussion

In thyroid cancer patients, NIS expression and I uptake are key factors for radionuclide therapy as I uptake is positively correlated with therapeutic response.^{5,19} From the patients at Seoul National University Hospital over the past 25 years, NIS protein expression has been observed in metastatic lesions by immunostaining and compared to immunostaining after ^{131}I therapy. Interestingly, approximately 80% of

patients positive for NIS immunostaining responded positively to ^{131}I therapy. These results indicate that thyroid cancer cells with high NIS expression uptake more ^{131}I , thus improving the response to ^{131}I therapy. However, 20% of NIS-positive patients did not respond to ^{131}I therapy, indicating that NIS expression cannot simply reflect the therapeutic efficacy of ^{131}I therapy in this group.²⁰

Because tumor response to the therapy varies at the single-cell level, tumor heterogeneity is considered an important factor for different therapeutic responses.²¹ Human cancers show substantial tumor heterogeneity in distinguishable phenotypic features, such as cellular morphology, gene expression, metabolism, and differential sensitivity to therapeutic agents and radiation.²² Although NIS expression in tumors is associated with the therapeutic response of ^{131}I in thyroid cancer patients, the level of NIS expression cannot precisely predict therapeutic outcome in some patients. In this research, we showed various apoptotic responses induced by ^{131}I in CMV-NIS (shown in Figure 2C, right). We also compared ^{131}I uptake from two different NIS-expressing tumors (one with high NIS and one with low NIS) and apoptosis imaging using ApoFlamma. From the analysis of ApoFlamma imaging in this study, the population of apoptotic cells in high NIS-expressing cells (CMV-NIS) was about 55% after ^{131}I treatment in vitro. In low NIS-expressing cells

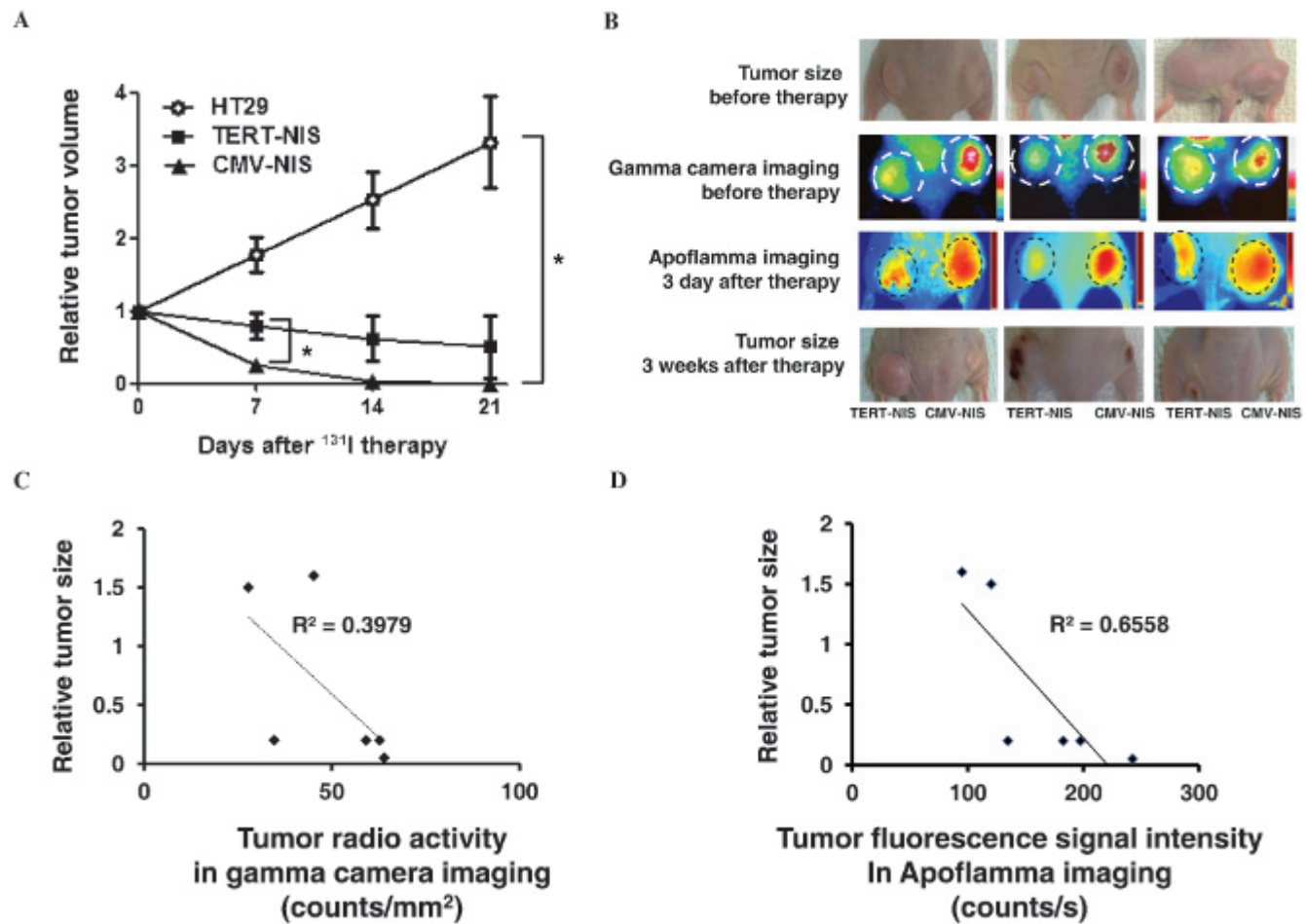


Figure 4. The correlation between gamma camera imaging, ApoFlamma imaging, and tumor size reduction. **A**, Tumor size was measured for 3 weeks after ^{131}I administration to evaluate the therapeutic effects. Tumor volume was significantly decreased in tumors with high NIS expression (CMV-NIS) than in those with low NIS expression (TERT-NIS) ($*p < .05$; $n = 3$). **B**, Gamma camera imaging and ApoFlamma imaging were compared to tumor size (following RECIST criteria) before and after ^{131}I therapy. Tumor size at 3 weeks after ^{131}I therapy was graphed with (C) radioisotope uptake and (D) ApoFlamma fluorescent signals. Relative tumor size indicates the ratio of tumor size (tumor size at 3 weeks/tumor size before ^{131}I therapy).

(TERT-NIS), the population of apoptotic cells was only 1% after ^{131}I treatment, confirming that I uptake was relatively low in cells with lower NIS expression. This result indicates that all NIS-expressing cells do not show the same level of ^{131}I -induced apoptosis under an equal amount of radiation exposure. Cells from either lower or higher NIS-expressing tumors showed different levels of apoptosis, and this result provides a clue to the different therapeutic responses to ^{131}I treatment in tumor cells expressing different levels of NIS.

NIS activity in NIS-expressing tumors was monitored with gamma camera imaging before ^{131}I therapy. It was reported that $^{99\text{m}}\text{Tc}$, ^{125}I , ^{131}I , and rhenium 188 (^{188}Re) share the same uptake mechanism through NIS protein, and uptake imaging signals of $^{99\text{m}}\text{Tc}$ and other radionuclides in the gamma camera imaging were highly

correlated between cell numbers, which can be used as an indicator of NIS activity.²³ A correlation between NIS activity and apoptosis was observed.

In this research, radioisotope uptake by NIS and apoptosis in the course of ^{131}I therapy was also compared to tumor reduction (shown in Figure 4). Through radioisotope uptake and apoptosis imaging at an early time point (3 days) after therapy, the therapeutic effect of ^{131}I treatment could be predicted much earlier than analyzing tumor size reduction, which can only be clearly monitored 3 weeks after therapy. However, tumor reduction was more correlated with apoptosis than radioisotope uptake through NIS. Therefore, apoptosis imaging by ApoFlamma can provide useful information to predict the therapeutic response to ^{131}I treatment at an early stage of tumor therapy, allowing consideration of other treatment

options for nonresponders. Evaluation of cell death through apoptosis is important to predict the precise therapeutic effects of ^{131}I treatment,^{24,25} and these less responding or nonresponding tumors for ^{131}I require additional therapeutic strategies, such as combination therapy including chemotherapy and radiotherapy.^{3,5,26} Standard protocols for thyroidectomized patients should wait for at least 6 months to know the therapeutic response of ^{131}I therapy.⁵ In this regard, if it is possible to predict the therapeutic effect of ^{131}I at an early stage of therapy, physicians have more time to prepare additional therapeutic options for nonresponding or less responding patients.

Limitations do exist in the current study. Apoptosis imaging using ApoFlamma cannot distinguish apoptotic cells and necrotic cells. ApoFlamma could be targeting histone H1 on the membrane of apoptotic cells and histone H1 in the nucleus of necrotic cells.²⁷ In contrast to apoptosis, necrosis evokes a strong inflammatory response and sometimes results in more aggressive tumors due to the stimulatory role of inflammation on tumor growth.²⁸ Alternatively, apoptosis is safe cell death as it does not cause an increase in toxic substances or local inflammation. Therefore, the ApoFlamma signal sometimes does not represent net apoptosis as a true indicator of successful therapy. In this regard, an efficient method is still required to be developed to distinguish between necrosis and apoptosis for evaluating the therapeutic response.²⁹ Another limitation of the study is in vivo application of fluorescent dye for imaging. Due to the limited tissue penetration depth of light for fluorescent dye, the fluorescent signal was used only for in vivo imaging. However, many near-infrared fluorescent dyes with improved penetration depth and a high signal to background ratio have recently been developed.³⁰ Since ApoFlamma used FPR-648 fluorescent dye and emits a relatively longer wavelength, our system could successfully demonstrate ^{131}I -mediated apoptosis in the NIS-expressing tumors in vivo. Successful radioisotope labeling was also reported, and both can be applied to overcome the limitation of fluorescence imaging.²⁷

Conclusion

In this study, apoptosis imaging using ApoFlamma visualized successful apoptosis induced by ^{131}I therapy in tumors expressing different levels of the NIS gene. Although the apoptotic response of each cell against ^{131}I therapy was quite heterogeneous, radioisotope uptake through NIS and apoptosis were positively correlated with

therapeutic responses of ^{131}I . These results indicate that apoptosis imaging can provide useful information to predict the therapeutic response to ^{131}I treatments.

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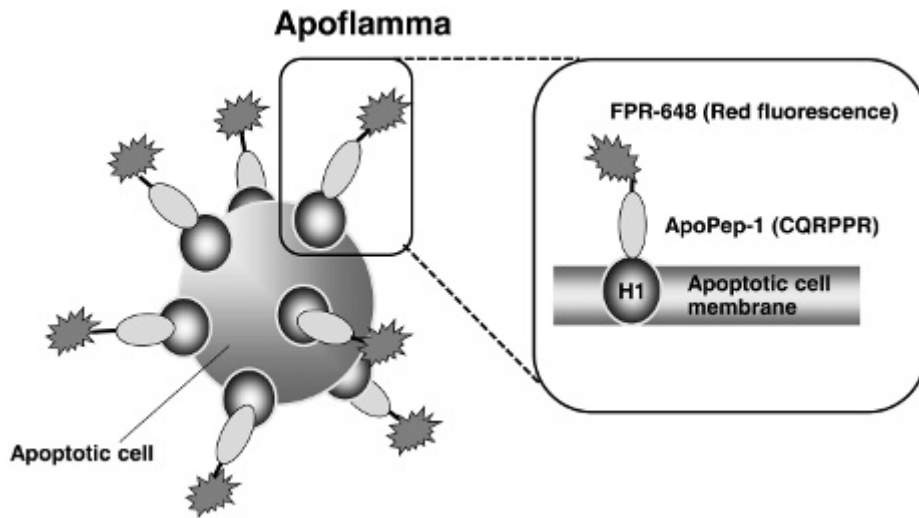


Figure S1. Diagram of binding ApoFlamma in apoptotic cells. ApoFlamma is a fluorescent dye (FPR-648) conjugated probe for apoptosis using histone H1 targeting peptide (ApoPep-1), which consists of six amino acids (CQRPPR). ApoFlamma binds to histone H1 exposed on the surface of apoptotic cell membrane and shows efficient tissue penetration and rapid clearance with low immunogenicity.

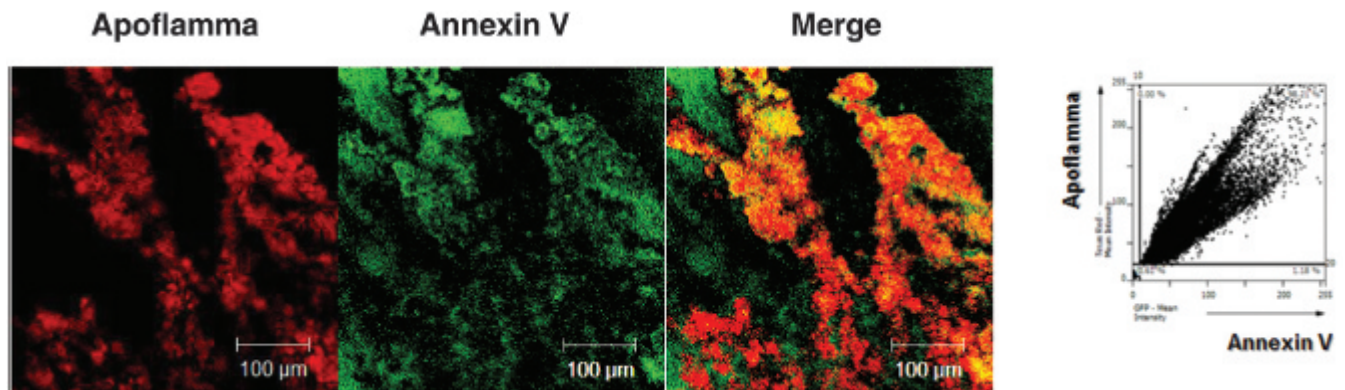


Figure S2. Comparison of apoptosis imaging between the ApoFlamma and annexin V administration on ^{131}I -treated tumor tissue (CMV-NIS). ApoFlamma and annexin V-fluorescein isothiocyanate (FITC) were performed to the tumor tissues 3 days after ^{131}I treatment (1.6 mCi). ApoFlamma targets nuclear membrane-localized histone H1, and annexin V targets phosphatidylserine at the inner membrane of cells.