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Characterization of somatostatin receptors (SSTRs) expression and antiproliferative effect of somatostatin analogues in aggressive thyroid cancers ☆

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ABSTRACT

Background: Certain human carcinomas have demonstrated a distinct expression of somatostatin receptors. Data on somatostatin receptor expression in follicular thyroid cancer and anaplastic thyroid cancer has been limited and conflicting. This study seeks to characterize somatostatin receptor expression in follicular thyroid cancer and anaplastic thyroid cancer and to assess the effects of somatostatin analogues.
Methods: Anaplastic thyroid cancer (Hth7 and 8505C) and follicular thyroid cancer (FTC-236) (Sigma-Aldrich, St. Louis, MO) cells were cultured. Capillary immunoblotting and reverse transcription polymerase chain reaction (RT-PCR) were used to determine the basal expression of protein and mRNA of SSTR1–SSTR5. Cells were treated with the somatostatin analogues octreotide, pasireotride (SOM230), and KE-108 for 48h. IC_{50} was determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, and cell proliferation was measured by viable cell count. Presence of SSTR2 was assessed by immunohistochemistry.
Results: Immunoblotting analysis demonstrated that most cell lines expressed SSTR1–SSTR3 and SSTR5 in varying degrees. Reverse transcription polymerase chain reaction analysis showed that mRNA expression for SSTR2 and SSTR3 correlated with protein expression. MTT assays showed that KE-108 and SOM230 were able to inhibit cell proliferation. Tissue microarray (TMA) showed that SSTR2 was highly expressed in human tissues of aggressive thyroid carcinomas.
Conclusion: Follicular thyroid cancer and anaplastic thyroid cancer express SSTR1-3 and SSTR5 in distinct fashions both at a message and protein level. Our results suggest that somatostatin receptors are still a relevant and promising drug target against non-medullary thyroid cancers.

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Introduction

With a rapidly increasing incidence around the world, thyroid carcinomas are the most prevalent endocrine malignancy.[1,2] Common therapies for these malignancies include treatment with radioactive iodine ablation, thyrotropin hormone suppressive therapy with thyroxin, or thyroidectomy. Still, approximately 20% of the patients will not respond well to therapy because thyroid malignancies de-differentiate and lose capacity for radioactive iodine uptake. Further de-differentiation limits therapeutic options and leads to poor prognosis.[3] Therefore, alternative treatments for these nonresponsive patients are needed.

Many carcinomas have been shown to express somatostatin receptors (SSTRs). Of these, SSTR2 is overexpressed commonly in endocrine tumors.[4] Furthermore, thyroid carcinomas are known to express SSTR2 and SSTR5. The presence of SSTRs in tumors provides the possibility of diagnostic imaging and therapy with radiolabeled SST analogs such as the FDA-approved analog octreotide and lutetium Lu-177 dotatate.[5,6] Thyroid tumors are known to express SSTRs distinctly depending on whether they are medullary thyroid cancer tumors or nonmedullary tumors: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), or anaplastic thyroid cancer (ATC).[7,8]

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FTC is a malignant epithelial tumor that accounts for 10%–32% of differentiated thyroid carcinomas.[1,9] Although it is uncommon, FTC has a greater rate of distant metastasis (29%) and can ultimately lead to death (17%) compared to PTC (9% and 8%).[10] ATC is the least common of all thyroid carcinomas (<1%). Still, ATC accounts for about 40% of thyroid cancer deaths. The mean survival rate of ATC is less than 6 months after diagnosis with a 5-year survival rate of 0–25%. Current aggressive, multimodal therapies fail to improve this survival rate.[11] Roughly 50% of patients exhibit metastasis to lungs (80%), bones (6%–15%), and brain (5%–13%). Therefore, there is a clear need for novel therapies for these aggressive thyroid cancers. The limited and conflicting data on SSTR expression for FTC and ATC hinders the application of somatostatin analogues as a potential treatment. Our study seeks to characterize SSTR expression in FTC and ATC and to assess the effects of somatostatin analogs in these thyroid carcinomas.

Materials and methods

Cell culture and reagents

Hth7 cells, an ATC cell line, were obtained from Dr Rebecca Schweppe (University of Colorado, Denver, CO). FTC-236, a metastatic FTC cell line, and 8505C, an ATC cell line, were purchased from Sigma Life Science/European Collection of Cell Cultures (Sigma-Aldrich, St. Louis, MO). All cell lines were authenticated and confirmed with their unique identity by DNA profiling.[11] Hth7 cells were maintained in glutamine (+) RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA) with 10% fetal bovine serum. FTC-236 cells were maintained in glutamine (+), DMEM:F-12 medium (Invitrogen Life Technologies) with 10% fetal bovine serum, 10 μg/ml insulin, and 10 mIU/ml of human thyroid stimulating hormone (Sigma-Aldrich). 8505C cells were maintained in glutamine (+) EMEM medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum and 1% non-essential amino acids (Sigma-Aldrich). All media were supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. All cell lines were grown in a humidified environment of 5% CO_2 at 37°C.

Octreotide (OCT), an SSTR2 specific agonist, was purchased from Sigma-Aldrich. Pasireotride (SOM230), an SSTR5, SSTR3>>SSTR2 agonist, and KE-108, a pansomatostatin receptor agonist SSTR1–SSTR5, were purchased from Biomatik USA (Wilmington, DE). OCT, SOM230, and KE-108 were dissolved in dimethyl sulfoxide and stored at −20°C as 50mM stock solutions.

Cellular proliferation assay

FTC-236, Hth7, and 8505C were treated with OCT, SOM230, and KE-108 in varying doses to determine the half-maximal inhibitory concentration (IC_{50}) of each compound using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each cell line was plated in quadruplicate on 96-well plates at a density of 5,000 cells per well. Plated cells were incubated overnight to allow cell attachment; treatment medium was prepared in serial dilutions for various concentrations of OCT (0–200 μM), SOM230 (0–200 μM), and KE-108 (0–128 μM). Each quadruplicate of cells was exposed to only 1 compound at a time. Quad Cell viability was measured 48 h after treatment. On the day of measurement, treatment medium was removed before cells were incubated at standard conditions in 25 μL of serum-free RPMI-1640 containing 0.5 mg/ml −1 MTT for 3 h. To dissolve the MTT formazan, 75 μL of dimethyl sulfoxide was added to each well and mixed thoroughly. Absorbance was measured at 562 nm using a spectrophotometer (μQuant; Bio-Tek Instruments, Winooski, VT). The IC_{50} value was calculated by GraphPad Prism 6 (GraphPad Software, La Jolla, CA).

Quantitative real-time PCR

Thyroid cancer cells were cultured 2 days before total RNA was isolated using an RNeasy Plus Mini Kit (Qiagen, Valencia, VA). Total RNA concentration was determined by NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Complementary DNA was synthesized from 2 μg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). The quantitative real-time PCR was performed in triplicate on MyiQ Thermal Cycler (Bio-Rad Laboratories). Target gene expression was normalized to S27 levels in respective samples as an internal standard, and the comparative cycle threshold method was used to calculate relative expression levels of target genes.

Capillary immunoblotting

Thyroid cancer cells were cultured 2 days before protein lysates were isolated, as described previously.[12] Protein lysates were prepared at a concentration of ~1.5 μg/μL and delivered to RayBiotech (Norcross, GA) for capillary immunoblotting. Target protein expression was normalized to beta-actin levels in respective samples as an internal standard. A densitometry analysis was carried out with the band intensity data provided by RayBiotech (Norcross, GA).

Human tissue microarrays

The human tissue microarrays consisted of 159 samples distributed as follows: 10 normal thyroid samples, 10 nodular goiter samples, 10 Hashimoto's samples, 32 adenoma samples, 57 PTCs, 28 FTCs, 2 poorly differentiated thyroid cancers, and 10 ATCs. SSTR2 expression was quantified within the nucleus and cytoplasm of each core with the Vectra 3.0 workflow as described previously using an anti-SSTR2 monoclonal antibody (Abcam, San Francisco CA; CAT#ab134152).[13] Total SSTR2 staining was used to stratify patients into low- and high-expression groups using the median of 0.15 as the cutoff. Spectral libraries were assembled using Nuance 3.0.0 and inForm 1.2 (Caliper Life Sciences, Hopkinton, MA).

Results

mRNA expression of SSTR1-5

To elucidate the expression of the SSTR1-5 isoform in ATC and FTC mRNA, we employed quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis (Fig. 1). There are varying degrees of mRNA expression for SSTR1, SSTR2, SSTR3, and SSTR5 among cell lines. SSTR1 mRNA was only detected in the FTC-236 sample (Fig. 1, *A*). SSTR2 mRNA (Fig. 1, *B*) was greatest in the ATC cells (8505C and Hth7) and least in FTC-236. SSTR3 mRNA (Fig. 1, *C*) was highly expressed in Hth7 and moderately expressed in all other cell lines. SSTR5 mRNA (Fig. 1, *D*) was detected in all cell lines and was greatest for FTC-236. SSTR4 mRNA was not detected in any of the cell lines.

Protein level expression of SSTR1-5

Western blots and densitometry analysis were used to characterize the protein expression of SSTR1-SSTR5 in ATC and FTC cell lines (Fig. 2). All thyroid cancer cell lines expressed SSTR1, SSTR2, SSTR3, and SSTR5 in varying degrees. SSTR3 was highly expressed through all cell lines; however, none of the cells analyzed expressed SSTR4 at a protein level.

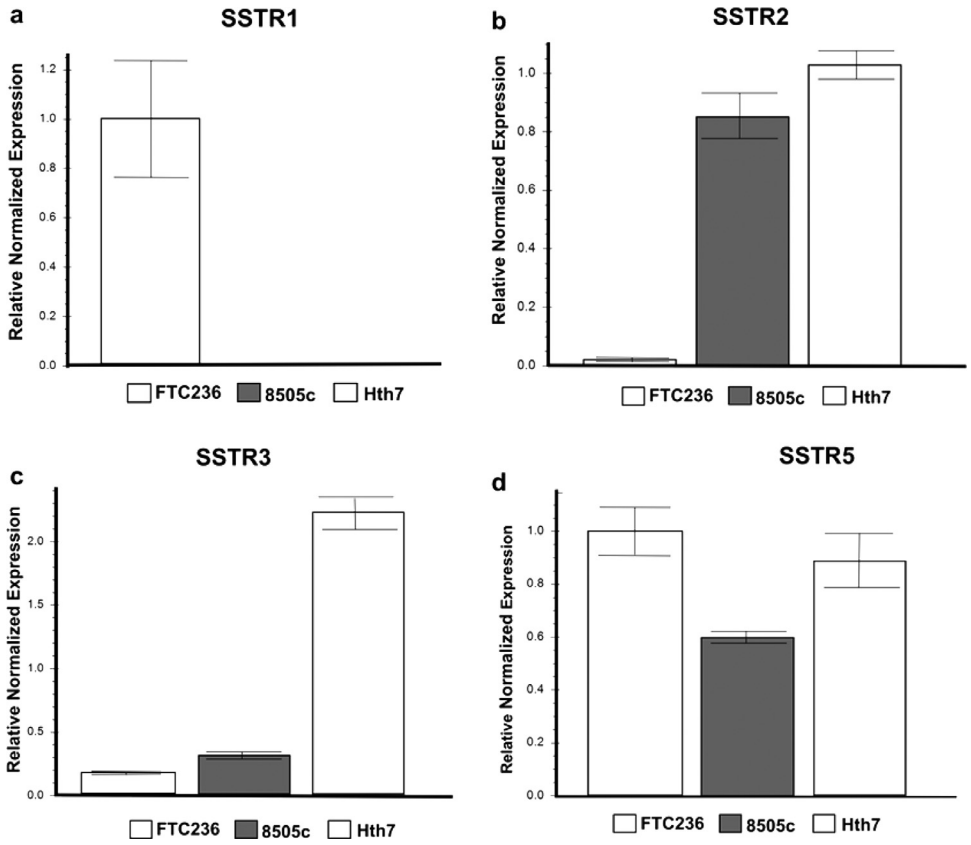

Fig. 1. mRNA expression of somatostatin receptors (SSTR1-SSTR5) varies between FTC and ATC cell lines. SSTR1 (A) is only expressed by FTC-236. SSTR2 (B), SSTR3 (C) and SSTR5 (D) are variably expressed by all cell lines.

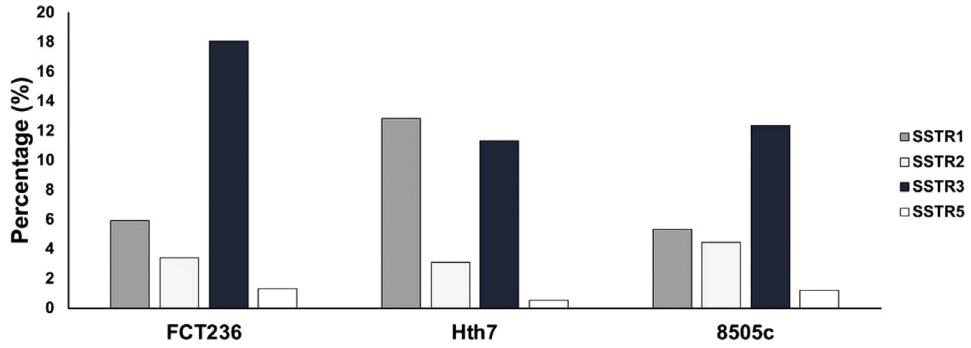

Fig. 2. Densitometry analysis was carried by normalizing target protein expression to betaactin levels. SSTR3 showed the highest expression of all SSTRs followed by SSTR1 > SSTR2 > SSTR5.

Antiproliferative effect of OCT, SOM230, and KE-108 in ATC and FTC cell lines

MTT assays were performed to determine the effect of somatostatin analogs on ATC and FTC cellular proliferation. ATC (Hth7, 8505C) and FTC (FTC-236) were treated with varying dosages of OCT (Fig. 3, *A*), SOM230 (Fig. 3, *B*), and KE-108 (Fig. 3, *C*). OCT, an SSTR2 specific agonist, showed little effect on cell growth inhibition with a less than 20% decrease in viability for all cell lines. Treatment with both SOM230, an SSTR5, SSTR3>>SSTR2 agonist, and KE-108, a pansomatostatin (SSTR1–SSTR5) receptor agonist, demonstrated effective and distinctive antiproliferative effects on malignant cell lines. Treatment with SOM230 was most effective on FTC-236 ($IC_{50} = 50\mu M$) followed by Hth7 ($IC_{50} = 55\mu M$) and 8505C ($IC_{50} = 90\mu M$). KE-108 treatment was most effective on 8505C ($IC_{50} = 30\ \mu M$) followed by Hth7 ($IC_{50} = 70\ \mu M$) and FTC-236 ($IC_{50} = 100uM$).

Human tissue microarrays

Tissue samples were formalin-fixed, paraffin-embedded, core thyroid biopsies mounted in triplicate (Fig. 4). The Vectra platform was employed to acquire images and measure SSTR2 staining intensities within the nuclear and cytoplasmic segments of each core. SSTR2 was absent in 10 normal thyroid tissues but present in 3 aggressive human thyroid cancers.

Discussion

There is an existing clinical challenge for the treatment of aggressive thyroid cancers, such as metastatic FTC and ATC.[1,14] FTC and ATC represent 19% and 1%–2% of all thyroid cancers, respectively. Coupling the poor differentiation of ATC with its frequent metastasis, however, accounts for over half of thyroid cancer–related deaths.[15,16] All patients with ATC are classified as having

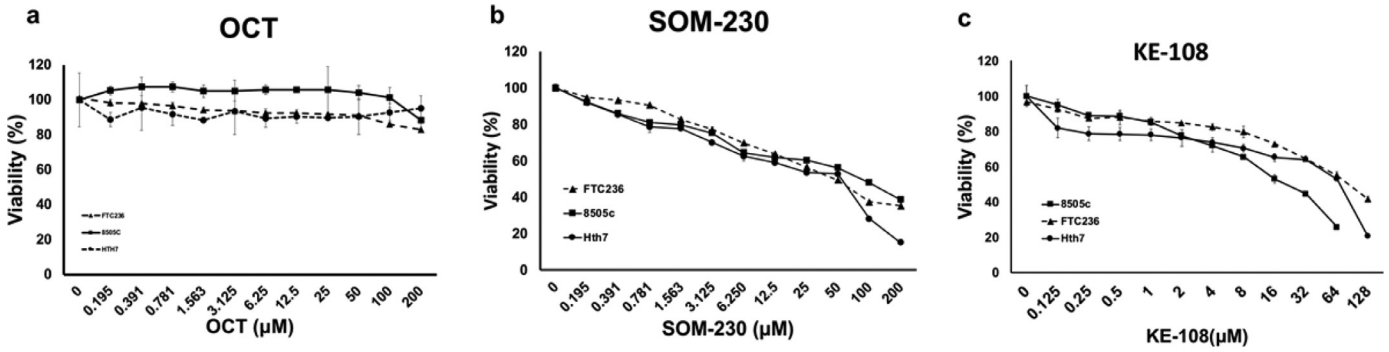

Fig. 3. Somatostatin analogs show varying effects on the viability of ATC (Hth7, 8505C), FTC and (FTC-236). Cells were treated with varying doses of OCT (0–200 μM), SOM-230 (0–200 μM) and KE-108 (0 – 128 μM) for 48 h. Cell viability was measured by the MTT assay. The data is presented in mean percent viability ± standard error of the mean (SEM). OCT (A) showed very limited cytotoxicity. SOM-230 (B) and KE-108 (C) showed effective and distinctive potencies in inhibiting malignant cell proliferation.

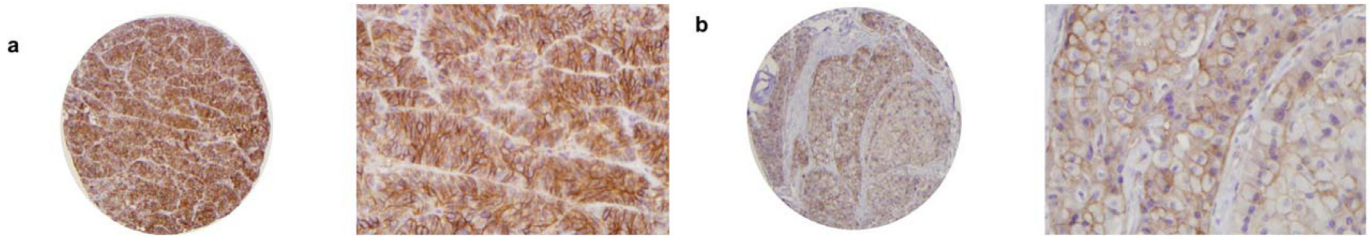

Fig. 4. SSTR2 was strongly expressed on (A) poorly differentiated thyroid cancer tissue and moderately expressed on a (B) PTC thyroid cancer tissue. No SSTR2 expression was found on normal thyroid samples.

a stage IV disease. Although most well-differentiated thyroid cancers can be cured via thyroidectomy and radioactive iodine ablation, approximately 20% of patients present a more aggressive phenotype with distant metastasis or recurrence associated with increased mortality.[17,18] Therefore, there exists a pressing need for alternative therapies for aggressive thyroid cancers.

Thyroid tumors are known to express SSTRs distinctively depending on whether they are medullary thyroid cancer or the non-medullary tumors, PTC, FTC, or ATC.[7,8] Current literature, however, suggests conflictive data on SSTR expression for the aggressive thyroid cancers ATC and metastatic FTC.

In an effort to unite these conflicting views, our study demonstrated that ATC and FTC cell lines expressed SSTR1, SSTR2, SSTR3, and SSTR5 in varying degrees. SSTR3 was highly expressed through all cell lines, followed by SSTR1, SSTR2, and SSTR5. None of the cells analyzed expressed SSTR4 at a protein or message level. Moreover, we found that there is a clear correlation between mRNA and protein expression for SSTR3, SSTR2, and SSTR5. We further attest the presence of a varied expression of SSTRs via the use of SST analogs OCT, KE-108, and SOM230. KE-108, a pansomatostatin receptor agonist, SSTR1–SSTR5, and pasireotride (SOM230), an SSTR5, SSTR3>>SSTR2 agonist, exhibited the best antiproliferative activity among these de-differentiated thyroid cancer cell lines. These results suggest that SST analogs could indeed be utilized as therapeutic agents for aggressive thyroid cancers. Although OCT was seemingly ineffective in our thyroid cancer cell lines, we cannot rule out its potential therapeutic efficacy against aggressive thyroid cancers given that our human thyroid tissues had substantial SSTR2 expression.

In conclusion, our results suggest that somatostatin receptor subtypes (SSTR1–SSTR3 and SSSTR5) are pertinent and potentially promising therapeutic targets for aggressive thyroid cancers. Our study is limited by the scarcity of both ATC and FTC cell lines as well as human thyroid samples of ATC and FTC. Further studies should look to characterize the role of SSTRs and somatostatin analogues on these aggressive thyroid cancers, for instance, to continue working on TMAs to further characterize expression of other SSTRs subtypes and to conduct biochemical assays to assess the inhibitory effects of SST analogues (eg, cell cycle arrest versus apoptosis). The characterization of the expression of SSTRs among thyroid cancer patients will permit a tailored, targeted therapy using radiolabeled peptides and improve patient survival rates.

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Conflicts of interest

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References

1. Schneider D, Chen H. New developments in the diagnosis and treatment of thyroid cancer. *CA Cancer J Clin*. 2013;63:373–394.
2. Haugen B, Alexander E, Bible K, Doherty G, Mandel S, Nikiforov Y, et al. 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: The American Thyroid Association guidelines task force on thyroid nodules and differentiated thyroid cancer. *Thyroid*. 2016;26:1–133.
3. Woelfl S, Bogner S, Huber H, et al. Expression of somatostatin receptor subtype 2 and subtype 5 in thyroid malignancies. *Nuklearmedizin*. 2014;53:179–185.
4. Klagge A, Krause K, Schierle K, et al. Somatostatin receptor subtype expression in human thyroid tumours. *Horm Metab Res*. 2010;42:237–240.
5. Pisarek H, Pawlikowski M, Marchlewska M, et al. An immunohistochemical investigation of the expression of somatostatin receptor subtypes—should therapeutic trials be performed to determine the efficacy of somatostatin analogs in treating advanced thyroid malignances. *Exp Clin Endocrinol Diabetes*. 2015;123:342–346.
6. Strosberg J, El-Haddad G, Wolin E, et al. Phase 3 trial of 177lu-dotatate for midgut neuroendocrine tumors. *N Engl J Med*. 2017;376:125–135.

7. Pazaitou-Panayiotou K, Janson ET, Koletsa T, et al. Somatostatin receptor expression in non-medullary thyroid carcinomas. *Hormones*. 2012;11:290–296.
8. Atkinson H, England JA, Rafferty A, et al. Somatostatin receptor expression in thyroid disease. *Int J Exp Path*. 2013;94:226–229.
9. Yang L, Shen W, Sakamoto N. Population-based study evaluating and predicting the probability of death resulting from thyroid cancer and other causes among patients with thyroid cancer. *J Clin Oncol*. 2013;31:468–474.
10. Ranganath R, Shah M, Shah A. Anaplastic thyroid cancer. *Curr Opin Endocrinol Diabetes Obes*. 2015;22:387–391.
11. Schweppe RE, Klopper JP, Korch C, Pugazhenthi U, Benezra M, Knauf JA, et al. Deoxyribonucleic acid profiling analysis of 40 human thyroid cancer cell lines reveals cross-contamination resulting in cell line redundancy and misidentification. *J Clin Endocrinol Metab*. 2008;93:4331–4341.
12. Somnay Y, Simon K, Harrison AD, Kunnimalaiyaan S, Chen H, Kunnimalaiyaan M. Neuroendocrine phenotype alteration and growth suppression through apoptosis by MK-2206, an allosteric inhibitor of AKT, in carcinoid cell lines in vitro. *Anticancer Drugs*. 2013;24:66–72.
13. Huang W, Hennrick K, Drew S. A colorful future of quantitative pathology: validation of Vectra technology using chromogenic multiplexed immunohistochemistry and prostate tissue microarrays. *Hum Pathol*. 2013;44:29–38.
14. Are C, Shaha AR. Anaplastic thyroid carcinoma: biology, pathogenesis, prognostic factors, and treatment approaches. *Ann Surg Oncol*. 2006;13:453–464.
15. Cornett WR, Sharma AK, Day TA, Richardson MS, Hoda RS, van Heerden JA, et al. Anaplastic thyroid carcinoma: an overview. *Curr Oncol Rep*. 2007;9:152–158.
16. Jang S, Yu XM, Odorico S, et al. Novel analogs targeting histone deacetylase suppress aggressive thyroid cancer cell growth and induce re-differentiation. *Cancer Gene Ther*. 2015;22:410–416.
17. Pacini F, Castagna MG, Brilli L, Pentheroudakis G. Differentiated thyroid cancer: ESMO clinical recommendations for diagnosis, treatment and follow-up. *Ann Oncol*. 2009;20:143–146.
18. Mazzaferri EL, Jhiang SM. Differentiated thyroid cancer long-term impact of initial therapy. *Trans Am Clin Climatol Assoc*. 1995;106:151–170.

Discussion

Dr Naris Nilubol (Bethesda, MD): A couple of questions: Regarding the level of expression of SSTR, have you looked at a positive control like neuroendocrine tumors? We know that neuroendocrine tumors often do respond to PRRT. How do they compare to neuroendocrine tumors? Do you have the data?

Number 2: From the cytotoxicity graph, the values of 100 micromolar, even 60 micromolar, would be a huge dose. Are these concentrations achievable in humans? Those are my 2 questions.

Ms Danilea M Carmona-Matos: Thank you for your questions. In my project in particular, we only focused on these anaplastic and follicular thyroid cancers, so I do not have data for other neuroendocrine tumors. However, I am aware that there is a differential response between neuroendocrine tumor versus anaplastic tumor.

In terms of the concentration, I'm not aware of the exact limits of using this drug in a human being right now. Perhaps we can find alternative drug delivery techniques. For example, if we use antibodies for the delivery, could we lower the dose? That is something that we look forward to exploring.

Dr Brian Untch (New York, NY): I think everybody is very interested in this kind of treatment because of FDA approval of Lutathera. I know Novartis has a number of trials outside of neuroendocrine tumors. So the comparison that was just talked about, I think, is really important so we can determine correlates of efficacy.

But my question is about your human tumors. How consistent was the SSTR expression by IHC and TMA? How many were positive, for example? How many were negative? Is this something that you see will have real clinical potential?

Ms Danilea M Carmona-Matos: Unfortunately, we had very limited tissue samples and cell lines with which to conduct this experiment. In terms of the positives for the poorly differentiated thyroid cancers, we had 3. We had about 30 samples of different aggressive thyroid cancers. We want to have more access to these tissues in order to better define clinical applicability.

Dr James Howe (Iowa City, IA): Nice study and good preliminary work. I'm just curious, since it's been around for a while, has anybody treated patients with anaplastic thyroid cancer with that, and is it a promising agent for imaging?

Ms Danilea M Carmona-Matos: We will hopefully be able to do translational work later on to determine if it's possible and if so, what would be the appropriate dosage.

Dr James Howe (Iowa City, IA): Has anybody tried it for imaging?

Ms Danilea M Carmona-Matos: I am not sure.

Dr Emad Kandil (New Orleans, LA): For your initial in vitro study, you used follicular and anaplastic thyroid cancer cell lines but there was no mention of any papillary thyroid cancer cell lines. Will you please clarify why you excluded aggressive PTC while you included FTC?

Ms Danilea M Carmona-Matos: The reason why we targeted only anaplastic and follicular tumors was because we knew there was a lot of work already conducted in PTC and we wanted to investigate these areas that were less understood. For example, many of the papers I reviewed were from 2000 to 2005 and perhaps only conducted RT-PCR, but not western blotting. We wanted to bring attention back to this area.

Dr Emad Kandil (New Orleans, LA): How did you decide to study SSTR2? Also, you mentioned that you used 3 aggressive PTC specimens while you excluded PTC from your in vitro studies. Can you please clarify what you meant by aggressive PTC? Also, please clarify how the scoring was performed by your pathologist.

Ms Danilea M Carmona-Matos: The reason why we used only SSTR2 for our immunohistochemistry is because upon our literature review, it was the one that was consistently expressed in prior work. It's something that we can find in neuroendocrine tumors as well as different types of thyroid cancers. We also found that of the antibodies that were previously used, it was the one that yielded more consistent results. Because of limited availability of samples, we wanted to concentrate on something we thought may work, if it was positive or not.

In terms of the aggressiveness of the tumors, our pathologist that conducted these experiments was Dr Lloyd in Wisconsin. I am not sure how he scored them because this was a pre-made TMA that he was providing us in collaboration.