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Award Number: W81XWH-04-1-0277

TITLE: Quantitative in situ Assessment of the Somatostatin Receptor in Breast Cancer to Assess Response to Targeted Therapy with 111-in-Pentetreotide

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REPORT DATE: May 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-05-2007	Final	1 Apr 2004– 30 Apr 2007

4. TITLE AND SUBTITLE

Quantitative in situ Assessment of the Somatostatin Receptor in Breast Cancer to Assess Response to Targeted Therapy with 111-in-Pentetreotide

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0277

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University
New Haven, CT 06520-8047

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

Somatostatin (SST) is a peptide hormone implicated in the growth and progression of cancers and SSTR2 is the predominant receptor subtype expressed in breast cancer. We hope to study the pattern of expression and clinical significance of SSTR2 levels in breast cancer. We have developed an algorithm called AQUA that can assess protein expression on tissue microarrays (TMA) based on molecular co-localization techniques. Our results show that SSTR2 is localized predominantly to the malignant cells although also in vessel/lymphatic elements. Although expression was not significantly correlated with survival on our TMA, it did appear to be overexpressed compared with benign breast tissue. A vessel compartment has been developed using a multiplexing protocol for co-localization of SSTR2 to tumor and endothelium concurrently. Cell line controls have also been developed as a normalization feature and ELISA assays have been more successful as reference protein measurements. Whole sections of breast cancer are currently being evaluated for SSTR2 expression and preliminary data are presented.

15. SUBJECT TERMS
breast cancer,quantitative analysis, tissue microarray, somatostatin

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	11	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

Somatostatin (SST) is a peptide hormone that inhibits the release of various hormones and growth factors. The receptors are also expressed in numerous tumors, with SSTR2, the predominant subtype expressed in breast cancer. Although there are some data for inhibitory effects of SST analogues in breast cancer, to date, small clinical trials of these agents have not been successful, perhaps in part because SST_R status prior to treatment was minimally investigated and varied in these studies. Until recently, SST_R expression has been performed by labor intensive methods such as autoradiography and RT-PCR *in vitro* and scintigraphy *in vivo*. We have developed a series of algorithms called AQUA that can assess protein expression on tissue microarrays (TMA) based on molecular co-localization techniques. Our automated analysis involves immunohistochemistry (IHC) combined with semi-automated acquisition and analysis of compartmentalized, quantitative, continuous scores which removes the inherent subjectivity of standard pathologist-based scoring systems. Our proposal further characterizes the expression and clinical significance of SST_R2 using large cohort breast cancer TMAs and outlines a means to translate and normalize the AQUA measurements from TMAs to whole section for clinical applications. In this manner, we hope to direct the development of targeted therapies to SSTRs more rationally.

Body

Task 1. Characterize SSTR2 expression in a breast cancer TMA

The initial goals of this aim have been completed however, further work expanding on the in situ measurements are being performed as outlined below.

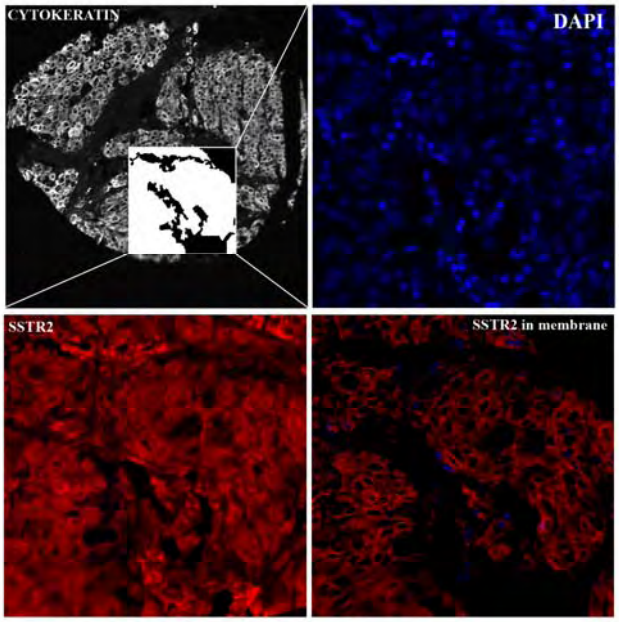
We have now completed a comprehensive evaluation of SSTR2 expression with several fold redundancy on a large cohort breast cancer tissue microarray (Figure 1). In summary, SSTR2 stained predominantly in the invasive tumors in a membranous pattern. This is consistent with other reports of SSTR2 expression in cancers (1-3). There also appeared to a lesser extent, variable levels in the stroma and vascular/lymphatic structures as well.

Outcome analysis showed that the standard markers such as tumor size, nodal status, and estrogen receptor, but not SSTR2 expression in the tumors were associated with disease-specific survival. Using X-tile (a statistical model developed for determination of optimal cutpoints of expression), the highest expressers were significantly associated with markers of poor prognosis (e.g. positive nodal status, large tumor size). This work has been presented at the DOD Era of Hope Breast Cancer Research Meeting June 2005.

In order to better compare SSTR2 expression in breast cancers versus normal breast epithelium, we also constructed and analyzed a TMA of normal breast tissue. These specimens were obtained predominantly from patients undergoing reduction mammoplasties. This showed again that SSTR2 stained predominantly within the membrane compartment of the epithelium but that the expression as a whole was substantially diminished compared to our breast

cancer cohort. This manuscript is currently being prepared for submission in the upcoming weeks (4).

Figure 1



Additionally, somatostatin and their interactions with somatostatin receptors on malignant cells and on endothelial cells have been hypothesized to play a role in the promotion of angiogenesis, either directly or via other proangiogenic factors such as vascular endothelial growth factor (VEGF). Indeed, in our arrays, SSTR2 expression on tumor cells was correlated with VEGF expression. Thus we have developed a model for creating a microvessel area (MVA) with AQUA using an endothelial marker such as CD31. Using a chicken keratin antibody and four channels, we can also simultaneously stain a slide with keratin (tumor), DAPI (nucleus), CD31 (endothelium), and a target antigen (e.g. VEGF, SSTR2), thus allowing quantitative co-localization of the target by AQUA to tumor or to endothelium (Figure 2). Our AQUA MVA when studied on the same breast cancer TMA showed that it was associated with larger tumors, node positivity, ER negativity, 20 year disease-specific survival at the univariate and multivariate levels, and with outcome (Kaplan-Meier survival curve Pvalue <0.0001) validating our MVA algorithms. These results have been recently submitted and is currently in review at Clinical Cancer Research (5). Towards our ultimate goal of correlating SSTR2 expression in microvessel and tumor compartments and correlating with analagous expression of downstream mediators of VEGF induced and/or somatostatin induced signaling in these compartments, we have acquired and performed initial experiments on breast test arrays for VEGF, VEGFR, AKT, pAKT, ERK, pERK, eNOS, and PI3kinase.

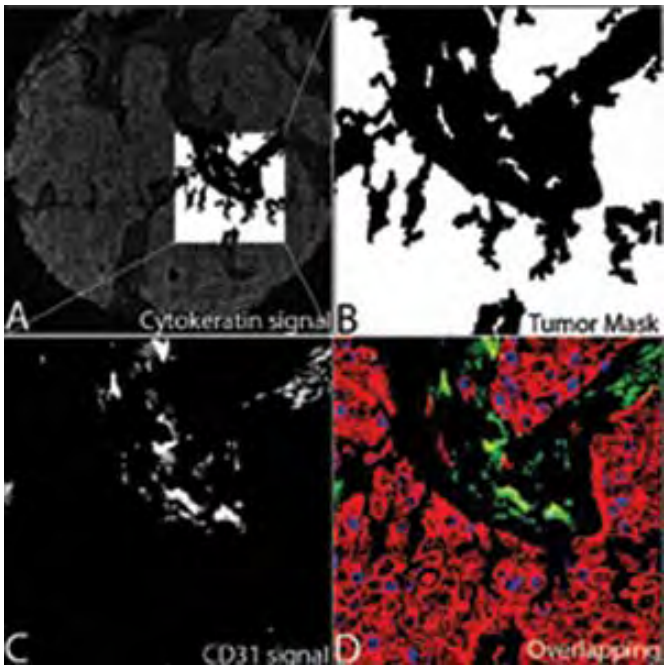


Figure 2

Task 2. Translating TMA-based AQUA algorithms to whole sections

Whole section analysis of ER from multiple slides/blocks of breast cancer has been completed and has recently been published (6). This showed good concordancy among blocks from the same specimen using AQUA or pathologist-based binary measurements but poorer concordancy when continuous AQUA scores were used.

The same breast cancer whole sections have now been stained with SSTR2 (same antibody used for TMA experiments) and AQUA analysis of the entire sections are currently being performed using similar methods to what was described in the above manuscript. Initial results suggest similar results to that seen with ER in that at approximately 5-10 images per section, there is a plateau in the variability of AQUA scores and that continuous measurements are more discordant than binary measurements (e.g. using the cutpoint determined by the Xtile system on the TMA for high versus low expressors) (7). We await the final analysis of this data.

Task 3. Conversion of AQUA to a protein concentration

In order to construct a standard curve for SSTR2 to serve as a reference point for conversion of AQUA scores of SSTR2 in tissue to protein concentration, we attempted to construct a serial dilution of SSTR2 peptide arrays in a matrix such as collagen. This however was quite problematic secondary to a variety of reasons, predominantly related to difficulties in constructing a peptide plug that was still stainable and readable by the AQUA protocol. We therefore have begun to use established breast cancer cell lines as a reference measure. 'Boutique' arrays are small arrays of 10-20 breast cancer cell lines processed into a cell line microarray (a procedure our lab has utilized in previous experiments involving harvesting the cell lines, formalin fixation, resuspension, then conventional paraffin embedding) have been constructed. An example of the range of AQUA scores

from these boutique arrays have been previously shown in last year's report. These arrays can then be added on to tissue arrays or whole sections adjacently on the same slide or at least stained side-by-side with the experimental slide. In this manner, slide-to-slide normalization is possible for comparative studies. In the future, we propose that all SSTR2 measurements with AQUA in tissue be performed with these cell line controls. Although ELISA measurements of these cell lines have also been somewhat difficult initially, we have now had better success with reproducibility as we have established a procedure in which cell lines are concurrently split for both boutique array construction as well as processed for other experiments such as ELISAs or western blots. We suspect this may minimize variability in tissue culture conditions that may lead to reproducibility problems.

Task 4. Study 111-In-pentetreotide activity and safety in patients with metastatic breast cancer

Unfortunately, since John Murren's (our co-investigator in this project) death, we have not been able to find a satisfactory co-investigator to replace Dr. Murren. The lack of Dr. Murren's extensive expertise in the somatostatin and radioactive peptide analogue fields will most likely make the completion of the important fourth and final task of this proposal not feasible at the current time. We have therefore requested a no-cost extension (status pending at this time) to complete the remainder of the unfinished tasks exclusive of task 4 and will then hopefully be able to recruit further support for the originally proposed trial of somatostatin analogue therapy in breast cancer.

Key Research Accomplishments

1. SSTR2 is expressed predominantly in the membrane compartment of breast tumor cells based on in situ AQUA measurements but is also present in stromal elements including apparent vessel compartments. Although SSTR2 is overexpressed in malignant breasts tumor cells compared with normal breast epithelial cells and was associated with several standard breast cancer prognostic parameters, it was not associated with survival.
2. An endothelial compartment can be constructed with AQUA using specific antibodies (e.g. CD31) that is prognostic of outcome and associated with breast cancer parameters (e.g. tumor size). This compartment can be used as another co-localization parameter such that quantitative measurements of SSTR2 within tumor and vessel compartments can be simultaneously obtained.
3. SSTR2 can be stained and analyzed by AQUA on whole tissue sections following the algorithms established by estrogen receptor. Grids of the whole sections can be placed ‘virtually’ on the section such that the entire section can be analyzed rapidly and efficiently. ER heterogeneity was most marked with continuous measurements and the preliminary data suggest similar results for SSTR2.
4. Boutique cell line arrays have become more readily accomplished. SSTR2 measurements in consistently designed boutique arrays are more consistent and more detailed SSTR2 ELISA measurements of these cell lines are ongoing.

Reportable Outcomes

1. QUANTITATIVE ANALYSIS OF SOMATOSTATIN RECEPTOR-2 ON A BREAST CANCER TISSUE MICROARRAY. Gina G. Chung, Sriparna Ghosh, Maciej P. Zerkowski, Robert L. Camp, David L. Rimm, John Murren. DOD (BCRP) Era of Hope Breast Cancer Research Meeting June 2005, Philadelphia, PA.

2. Characterization of somatostatin type 2 receptor expression in bone and soft tissue sarcomas. Ahrens W, DiCaprio M, Magit D, Chung G, Kellogg R, Lindskog D, Modlin I, Friedlaender G, Murren J. CTOS Annual Meeting 2005. Connecticut Tissue Oncology Annual Meeting, November 2005, Abstract 371, Boca Raton, FL.

3. Ghosh S, Zerkowski M, Camp RL, Rimm DL, Murren J, Chung GG. Quantitative analysis of somatostatin receptor-2 in breast cancer. Manuscript in preparation.

4. Ghosh S, Sullivan CAW, Ocal IT, Camp RL, Rimm DL, Chung GG. Microvessel area using automated image analysis is reproducible and is associated with prognosis in breast cancer. Manuscript in review, Clinical Cancer Research.

5. Chung GG, Zerkowski MP, Ghosh S, Camp RL, Rimm DL. Quantitative analysis of estrogen receptor heterogeneity in breast cancer. Laboratory Investigation, advance online publication 5 March 2007.

Conclusions

We have begun a systematic analysis of the expression of the SSTR2 in breast cancer using our automated analysis methodology which allows rapid, reproducible, quantitative measurements of in situ protein expression on tissue arrays. Our results show that SSTR2 is expressed in a graded fashion in a large proportion of breast cancers, is expressed predominantly within tumors and less so in stromal elements, and that it is mostly expressed in the membrane compartment of tumors. Although expression was not significantly correlated with survival on our TMA, it did appear to be significantly overexpressed in malignant breast epithelium compared with benign breast tissue. These results have now been reproduced in multiple fold, large cohort TMAs with several different antibodies. Because SSTR2 have been implicated in tumor angiogenesis and because our initial results suggested SSTR2 localized to tumor associated microvessels, we have also initiated work on creating a vessel compartment with AQUA. This has been readily accomplished using similar AQUA algorithms and a CD31 antibody and shows that an AQUA-based microvessel area is feasible, that it is associated with survival and other prognostic parameters in breast cancer, and that co-staining with other markers (e.g. VEGF, SSTR2) using a multiplexing protocol is feasible. Furthermore, cell line controls have been developed into "boutique array" with known relative levels of SSTR2 to serve as inter-slide normalization measures. Using ER as a prototype biomarker in breast cancer, we have translated the AQUA methodology to whole sections and work is now underway to assess SSTR2 in whole breast cancer sections and to determine what the relative SSTR2 heterogeneity in breast cancer is.

References

1. Janson ET, Stridsberg M, Gobl A, et al. 1998 Determination of somatostatin receptor subtype 2 in carcinoid tumors by immunohistochemical investigation with somatostatin receptor subtype 2 antibodies. Cancer Res 58:2375-2378
2. Kimura N, Pilichowska M, Date F, et al. 1999 Immunohistochemical expression of somatostatin type 2A receptor in neuroendocrine tumors. Clin Cancer Res 5:3483-3487
3. Reubi JC, Kappeler A, Waser B, et al. 1998 Immunohistochemical localization of somatostatin receptors sst2A in human tumors. Am J Pathol 153:233-245
4. Ghosh S, Zerkowski M, Camp RL, Rimm DL, Murren J, Chung GG. Quantitative analysis of somatostatin receptor-2 in breast cancer. Manuscript in preparation.
5. Ghosh S, Sullivan CAW, Ocal IT, Camp RL, Rimm DL, Chung GG. Microvessel area using automated image analysis is reproducible and is associated with prognosis in breast cancer. Manuscript in review, Clinical Cancer Research.
6. Chung GG, Zerkowski MP, Ghosh S, Camp RL, Rimm DL. Quantitative analysis of estrogen receptor heterogeneity in breast cancer. Laboratory Investigation, advance online publication 5 March 2007.
7. Camp RL, Dolled-Filhart M, Rimm DL. X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. Clinical Cancer Research. 10(21):7252-9, 2004 Nov 1.