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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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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. To this end, 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 variably expressed in a large proportion of breast cancers and is localized predominantly to the malignant cells. Although expression was not significantly correlated with survival on our TMA, it did appear to be overexpressed compared with benign breast tissue. Cell line controls have been developed as a normalization feature and the AQUA algorithms have been translated to whole sections. We have also assessed multiple methodologies for SSTR2 expression in soft tissue/bone tumors and plan to pursue similar strategies in breast cancer.						
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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 SSTR status prior to treatment was minimally investigated and varied in these studies. Until recently, SSTR 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. We propose to further characterize the expression and clinical significance of SSTR₂ using large cohort breast cancer TMAs and to correlate *in situ* tissue measurements by AQUA with other measures of SSTR2 expression. In this manner, we hope to direct the development of targeted therapies to SSTRs more rationally.

Body

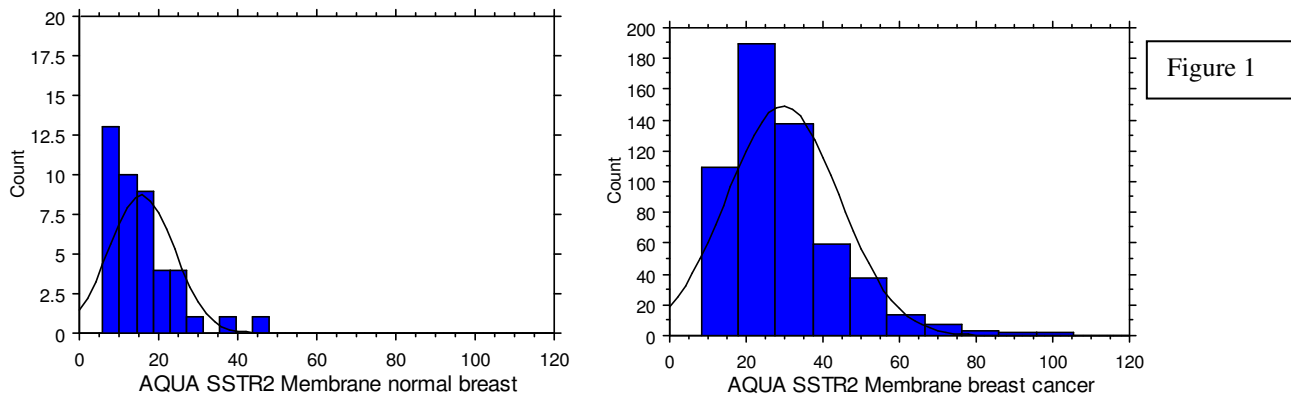
Task 1. Characterize SSTR expression in a breast cancer TMA

Since our last report, we have constructed two more redundant arrays with the same cases. We have collected extensive clinical and pathologic data on these patients, including disease-specific and overall survival. SSTR2 antibody (Santa Cruz, Carpinteria, CA) was obtained and initially titered on a breast cancer test array (TMA but with much fewer spots and without linkage to clinical information) to determine an optimal dilution. The full cohort TMA with 667 cases was then stained with SSTR2 and analyzed with AQUA. 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 (Figure 1). We have in the interim also tested another antibody to SSTR2 from Novus Biologicals and have demonstrated very similar staining patterns and clinical outcome correlations. Thus, although in our cohort of patients, SSTR2 expression did not correlate significantly with disease-specific outcome, the clear overexpression of SSTR2 in tumors and the predominant tumoral rather than stromal localization suggest that future studies of SSTR2 as a homing target for labeled somatostatin analogues may be an effective strategy.



Additionally, somatostatin and their interactions with somatostatin receptors on malignant cells and on endothelial cells/endothelial precursor cells have been hypothesized to play a role in the promotion of angiogenesis, either directly or via other proangiogenic factors such as VEGF. Thus we are also developing a model for creating a vessel mask 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). Towards this end, we have acquired and performed initial titrating experiments on breast test arrays for VEGF, VEGFR, AKT, pAKT, ERK, pERK, eNOS, and PI3kinase as downstream mediators of VEGF induced and/or somatostatin induced signaling.

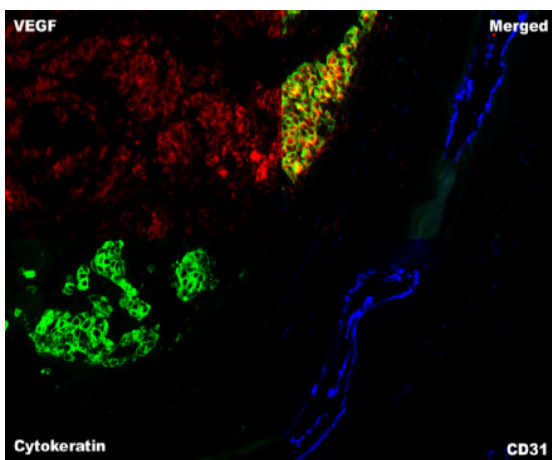
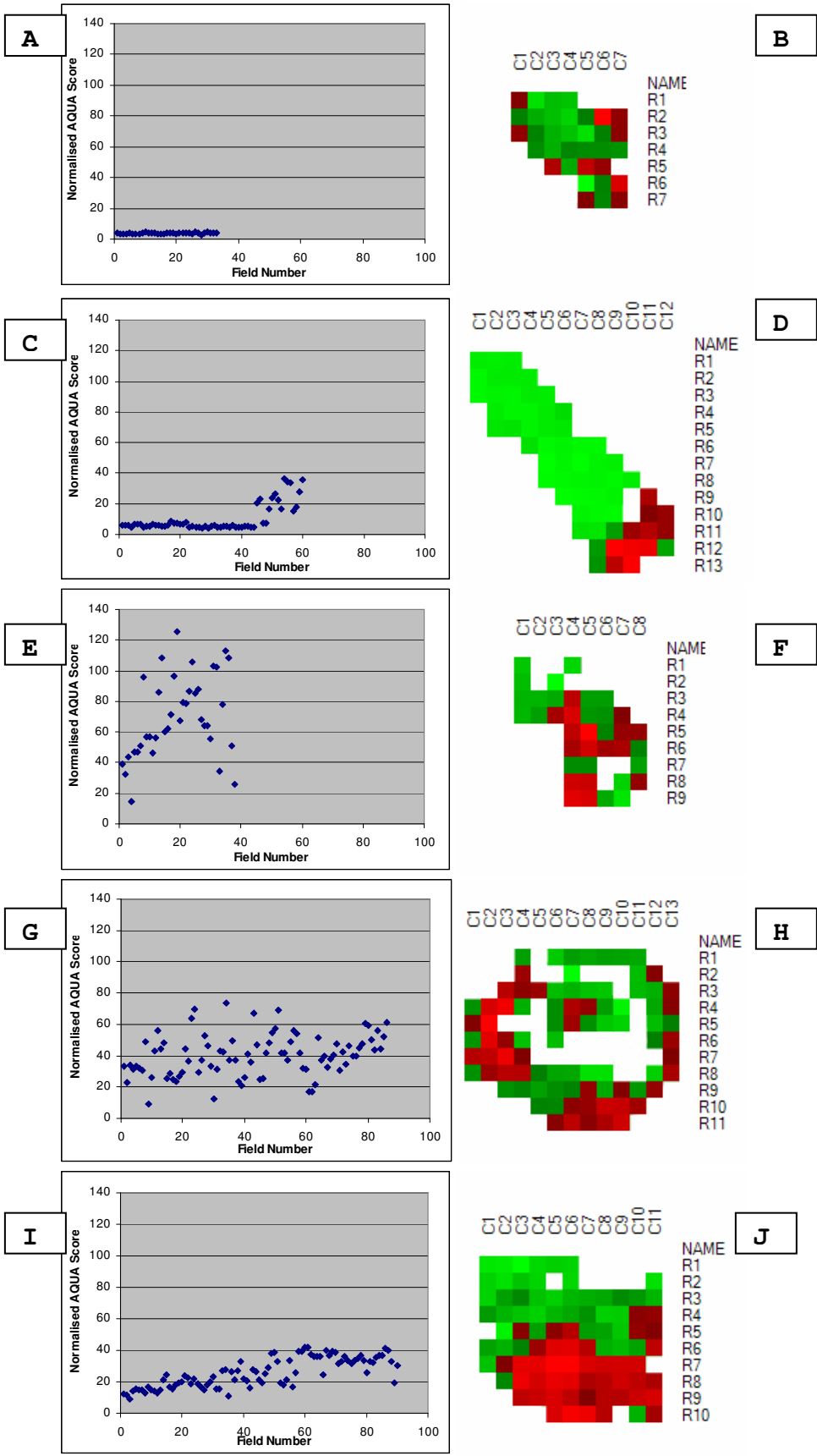


Figure 2

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

We have been doing initial studies for this task with estrogen receptor (ER) on whole tissue sections because this is a well characterized marker in breast cancer in which a pathologist-based "gold standard" exists. Multiple slides/blocks of breast cancer from the same patient were obtained from 11 cases of primary breast cancer and AQUA was used to quantify ER expression on multiple fields from each slide (over 2000 images). Our normalized ER scores ranged from 2.959 to 174.672. Most of the slides with low AQUA scores (<10) were relatively tightly clustered with minimal variance (Figure 3A). However, as the scores on a given slide increased, the variance generally increased (Figure 3E, 3G, and 3I). This finding did not seem to be strictly related to the number of fields analyzed per slide as high variance was seen with high number of fields (Figure 3G) as well as with low number of fields (Figure 3E). Corresponding 2-D "heat maps" were also generated based on the normalized AQUA scores (above mean score-red; below-green). Although for most slides, high and low scores on a given section appeared to be randomly scattered and with a normal distribution throughout the tumor (Figure 3B, 3F, and 3H), several slides showed a clustered pattern (Figure 3D and 3J). Interestingly, this clustering was seen in low scoring "ER negative" cases (Figure 3C and 3D) as well as in higher scoring "ER positive" cases (Figure 3I and 3J). Indeed, scattered as well as clustered patterns were seen even on different blocks from the same case (Figure 3G and 3H versus 3I and 3J).

Figure 3



Unpaired t-test comparisons showed that overall inter-slide concordancy was 19% and that the percentage of concordant cases (cases in which >50% of slide-to-slide comparisons were not significantly different) was 22% (Table 1).

Table 1. Slide-to-slide ER heterogeneity

Case	Block	Pathologist (%)	Mean AQUA score*	Standard Error	P value; Block Comparisons
A	1	0	3.667	0.074	0.2446; 1 vs 2
	2		3.461	0.152	
B	1	10	3.332	0.072	<0.0001; 1 vs 2
	2		2.911	0.039	<0.0001; 2 vs 3
	3		2.51	0.048	<0.0001; 1 vs 3
C	1	20	51.803	1.105	<0.0001; 1 vs 2
	2		25.884	0.976	<0.0001; 1 vs 3
	3		21.433	0.733	<0.0001; 1 vs 4
	4		40.12	1.444	0.0077; 2 vs 3 <0.0001; 2 vs 4 <0.0001; 3 vs 4
D	1	20	100	2.55	<0.0001; 1 vs 2
	2		40.978	3.71	
G	1	80	6.356	0.228	<0.0001; 1 vs 2
	2		10.063	0.289	0.0018; 1 vs 3
	3		5.527	0.138	<0.0001; 1 vs 4
	4		8.105	0.225	<0.0001; 1 vs 5
	5		4.979	0.167	<0.0001; 2 vs 3 <0.0001; 2 vs 4 <0.0001; 2 vs 5 <0.0001; 3 vs 4 0.1023; 3 vs 5 <0.0001; 4 vs 5
H	1	90	63.836	3.31	<0.0001; 1 vs 3
	2		57.639	1.935	0.0811; 1 vs 2
	3		45.614	2.466	0.0007; 2 vs 3
I	1	95	20.845	1.027	0.2047; 1 vs 2
	2		18.306	1.756	
J	1	100	10.151	1.162	0.0042; 1 vs 2
	2		7.035	0.366	0.0117; 1 vs 3
	3		6.93	0.285	0.69; 1 vs 4
	4		10.614	0.797	0.9348; 2 vs 3 0.0025; 2 vs 4 0.0067; 3 vs 4
K	1	100	36.778	2.056	<0.0001; 1 vs 2
	2		69.955	4.36	

* Normalised score to tissue controls and to maximum score (case D1)

Figure 4 shows slide-to-slide comparisons matched against the signout pathologist's score. Similar to the intra-slide assessment, inter-slide differences appear to be minimized with the lowest scores. Most notable however, are the discordancies between the pathologist and AQUA for cases

G, I, and J. Although we attempted to retrieve the original ER slides assessed by the signout pathologist for these cases, we were only able to locate Case I. This showed that the tumor was indeed diffusely and fairly homogenously positive, however the intensity appeared very weak. Because AQUA gives the average signal intensity in all pixels in a given area, it is possible that this may have accounted for the discordancy in this case.

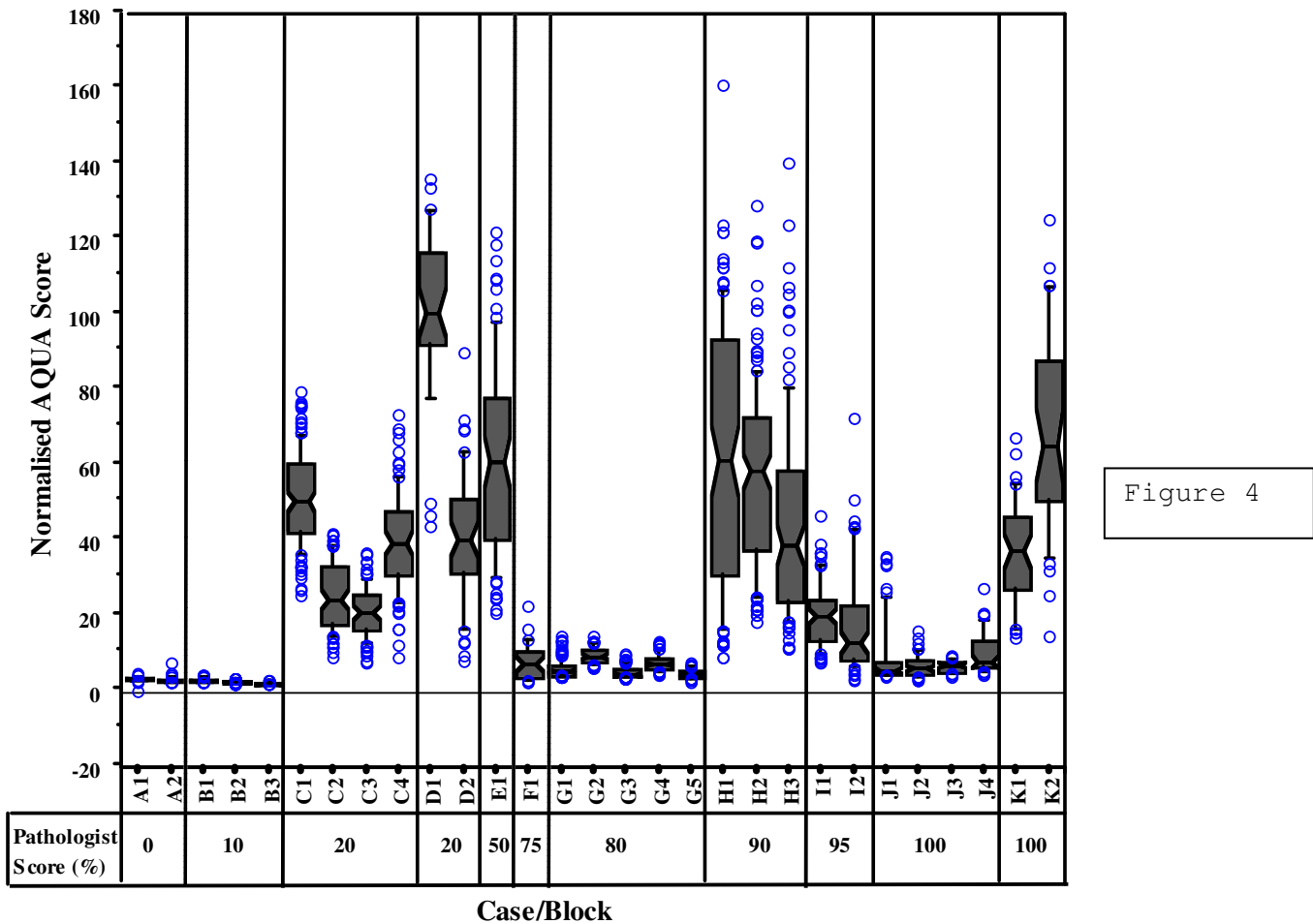


Figure 4

Our concordancy rate appears quite poor compared with previous studies looking at different assays for ER in the same tumor, ER assessments in matched core biopsies and surgical resections, and comparative ER levels in matched primary tumors and their metastases. However, prior studies of ER expression predominantly used binary categories, positive or negative, even when semi-quantitative methods such as the Allred score was used. Using a highly quantitative method such as AQUA, it is not surprising that more subtle differences missed by manual readings may now be detected. This may be important because higher levels of ER expression both by the ligand binding method and by semi-quantitative IHC readings has been associated with a greater liklihood of endocrine therapy response. However, other potential prognostic and predictive biomarkers (such as SSTR2) may more heavily rely on continuous readouts and more accurate assessments of total tumor heterogeneity. These data are currently being prepared for manuscript submission.

Task 3. Conversion of AQUA to a protein concentration

AQUA scores of cell blocks for SSTR2 are shown in figure 5. These lines were processed into a cell line microarray using a technique involving fixation, resuspension and pelleting only, and then paraffin embedding. This redundant cell line array can be either embedded into the large cohort TMA for simultaneous analysis of SSTR2 or constructed into a separate "boutique array" that can be stained and analyzed side-by-side with the breast cancer TMA, thus allowing for inter-slide normalization and valid inter-slide comparisons. The AQUA scores for these cell lines were quite variable and had a fairly wide range (nearly the range of AQUA scores seen in the tumor specimens on the TMA).

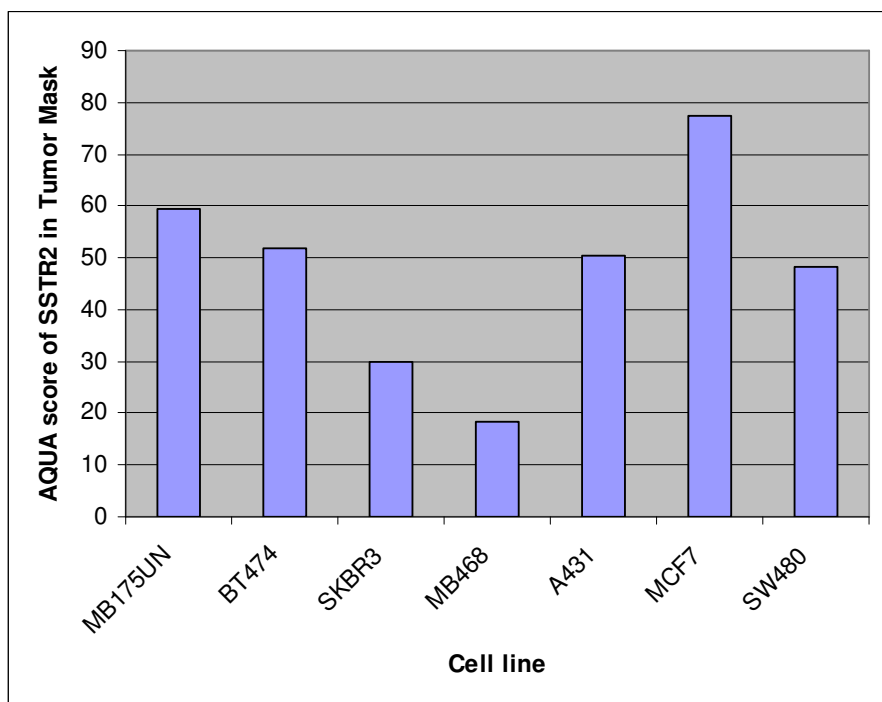


Figure 5.

As expected, MCF7 had high tumor mask and membrane expression (strong RT-PCR signal for SSTR2). We have found however, that the absolute quantification of protein in this manner to be quite problematic. Even AQUA analysis of cell blocks are difficult to reproduce with small variance, perhaps related to minor differences in cell culture conditions during cell block preparations. Whereas, this relatively small variability is relatively inconsequential for slide-to-slide normalization of AQUA scores, correlation with absolute protein concentrations by ELISA have been difficult to reproduce. We plan to study this further, although we feel that a method for inter-slide normalization of signal remains a valuable tool in of itself.

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

Our collaborative study of SSTR2 expression in soft tissue and bone tumors with co-investigator John Murren has preliminarily been concluded and presented in abstract form at the Connecticut Tissue Oncology Society Annual Meeting 2005 (Please see reportable outcomes). This study recruited

patients with sarcomas having definitive resections at Yale New Haven Hospital and compared SSTR2 expression by three different methodologies, RT-PCR on resected tissue, immunohistochemistry on resected specimen, and pre-surgery scintigraphy with ¹¹¹In-pentetreotide (Octreoscan). Thirty eight patients have been enrolled and analyzed in this preliminary analysis. Of the evaluable cases, this showed that 10/12 (83%), 29/30 (97%), and 30/36 (83%) were SSTR2 positive by scintigraphy, RT-PCR, and immunohistochemistry respectively. These results indicate that SSTR2 is present with high frequency in soft tissue and bone tumors, that analogues can be used to detect presence, and that there appears to be good correlation between scintigraphy and IHC.

Unfortunately, since Dr. Murren's death, this protocol (Dr. Murren was the P.I.) and the amendment to include breast cancer patients have been suspended. We are currently working with the Yale protocol review committee and the human investigations committee to open a separate protocol of similar design but uniquely for breast cancer. This protocol and consent form when ready for formal submission to the Yale IRB, will also be first forwarded to the DOD.

Key Research Accomplishments

1. Construction of redundant breast TMAs
2. Titrations of SSTR2 antibodies on test arrays
3. Correlation of SSTR2 expression on a large cohort breast cancer TMA with clinico/pathologic parameters
4. Construction of normal breast TMA and testing SSTR2 on normal breast TMA
5. Completion of the conversion of the AQUA algorithms from TMAs to whole tissue sections with estrogen receptor as a model
6. Validating SSTR2 antibodies on cultured cell lines, design of boutique arrays, and procedure for inter-slide normalization of AQUA scores
7. Clinical protocol studying SSTR2 expression by three different methodologies in patients with sarcomas or breast cancers

Reportable Outcomes

1. DOD Era of Hope Breast Cancer Research Meeting June 2005: QUANTITATIVE ANALYSIS OF SOMATOSTATIN RECEPTOR-2 ON A BREAST CANCER TISSUE MICROARRAY
2. Connecticut Tissue Oncology Annual Meeting, 2005: Characterization of somatostatin type 2 receptor expression in bone and soft tissue sarcomas

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 not stroma, 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. Furthermore, cell line controls have been developed into "boutique array" with know 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 methododlogy to whole sections. In the immediate future, we hope to analyze in a clinical study in vitro, in situ, and in vivo measurements of SSTR2 in breast cancer.

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