Expression of Somatostatin Type-2 and -4 Receptor and Correlation with Histological Type in Breast Cancer

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Abstract. *Background: Somatostatin is produced by hypothalamic cells and also by tumors. We were interested to evaluate the somatostatin type 2 (SSTR2) and type 4 (SSTR4) receptor expression on a large sample cohort of breast cancer cases. Materials and Methods: We used two different Tissue Micro Arrays (TMA) to evaluate SSTR2 and SSTR4 distribution. We evaluated the correlation between SSTR2 and SSTR4 expression and 18 tumor cells markers. We also assessed SSTR mRNA expression on an independent breast cancer population and correlated levels of SSTR2 and SSTR4 expression to molecular breast cancer subtypes. Results: 268 tumors were analyzed. The tumor overexpression of estrogen receptor was significantly correlated to the expression of SSTR2 (p=0.05) and SSTR4 (p=0.04). On principal component analysis, SSTR2 subtype characterized the luminal tumor type. On an independent breast cancer population, expression of SSTR2 and SSTR4 are independent from Human Epidermal Growth Factor Receptor 2 (Her2) and correlated with luminal tumors. Conclusion: Expression of somatostatin receptors is a marker of luminal breast tumors.*

Somatostatin is an hormone produced by hypothalamic cells, neurons, delta cells of the stomach, intestine and pancreas but also by tumor cells. Initially secreted as a long precursor molecule, it undergoes specific enzymatic degradation generating two types of somatostatin: somatostatin 14 (14 amino acids) and somatostatin 28 (28 amino acids). Both peptides are produced in different proportions depending on the tissue and regulate hormone secretion at different levels of the body (1). Two main biological effects of somatostatin are described in literature: anti-tumoral (2-5) and anti-secretory (1, 6-8).

There are five different types of somatostatin receptors (SSTR) named SSTR1 to SSTR5. These transmembrane receptors are coupled to G-proteins and each specific gene is located on different chromosome letting suggest a specific function for each of them (9). Since the development of polyclonal antibodies specific to the SSTR subtype, we could specify the location of each SSTR and evaluate their proportion in healthy and pathological tissues (10-12). SSTR expression is variable depending on cell type. In normal tissues, SSTRs are found primarily in brain, pancreas, stomach and kidney. Distribution in tumor is variable depending on the tumor type and biological characteristics (1, 9).

In breast cancer, the five SSTR subtypes are expressed with predominance for SSTR2 (SSTR1: 91%, SSTR2: 98%, SSTR3: 96%, SSTR4: 76% and SSTR5: 54%) (13). In addition, it has been shown that more than 50% of breast tumors express SSTR and were more often present in well-differentiated tumors and hormone receptor-positive tumors (14-16).

The aim of the present study was to evaluate SSTR2 and SSTR4 expression on a large sample of breast cancer using immunohistochemistry on breast tissue micro array (TMA) and correlate those expressions with biological tumor characteristics.

Materials and Methods

Human breast tissues. We used two different TMA to evaluate SSTR2 and SSTR4 distribution. The first involved 105 patients aged under 43 years' old. For each of them, 3 slices of invasive tumor and one of normal breast tissue were sampled. The second one involved 100 patients, half of them had unifocal tumor and the others had multifocal tumor. For each patient, 3 slices of

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Key Words: Breast cancer, somatostatin receptors, immunohistochemistry, luminal tumor.

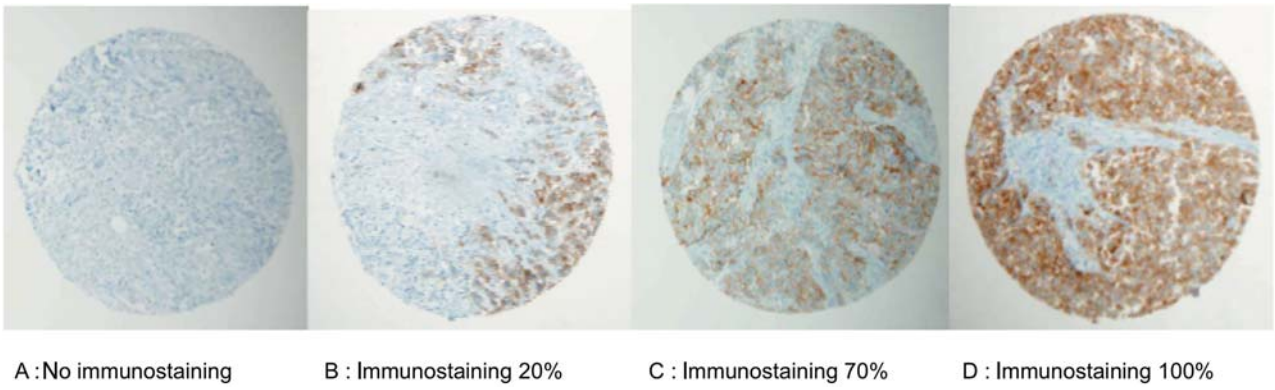

Figure 1. *Samples of SSTR2 immunostaining on TMA breast cancer tissues.*

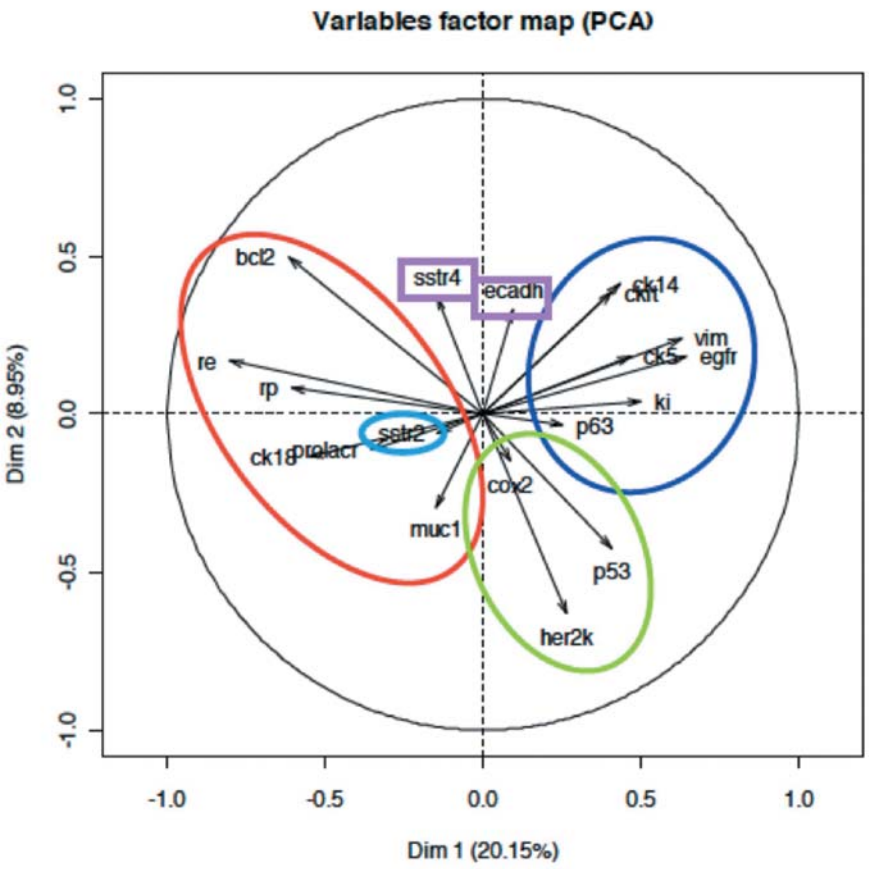

Figure 2. *Principal component analysis for SSTR2 and SSTR4.*

invasive tumor and 2 of normal breast tissue were sampled. We included placenta, bronchial tissue and prostate as control tissue.

Antibodies. To detect SSTR2, a commercially-available anti-SSTR2A polyclonal antibody, RBK046-05 (Zytomed Systems, Berlin, Germany®) was used at a dilution 1:100). It has previously been tested and developed on endocrine pancreas cells (17-20).

To detect SSTR4, a commercially-available anti-SSTR4 polyclonal antibody (Chemicon International®, Billerica, MA, USA) was used at a dilution 1:400).

Immunohistochemistry. The immunohistochemical procedures were performed as previously described (21). Three μm thick sections were cut from the TMA paraffin blocks and mounted. First, sections were

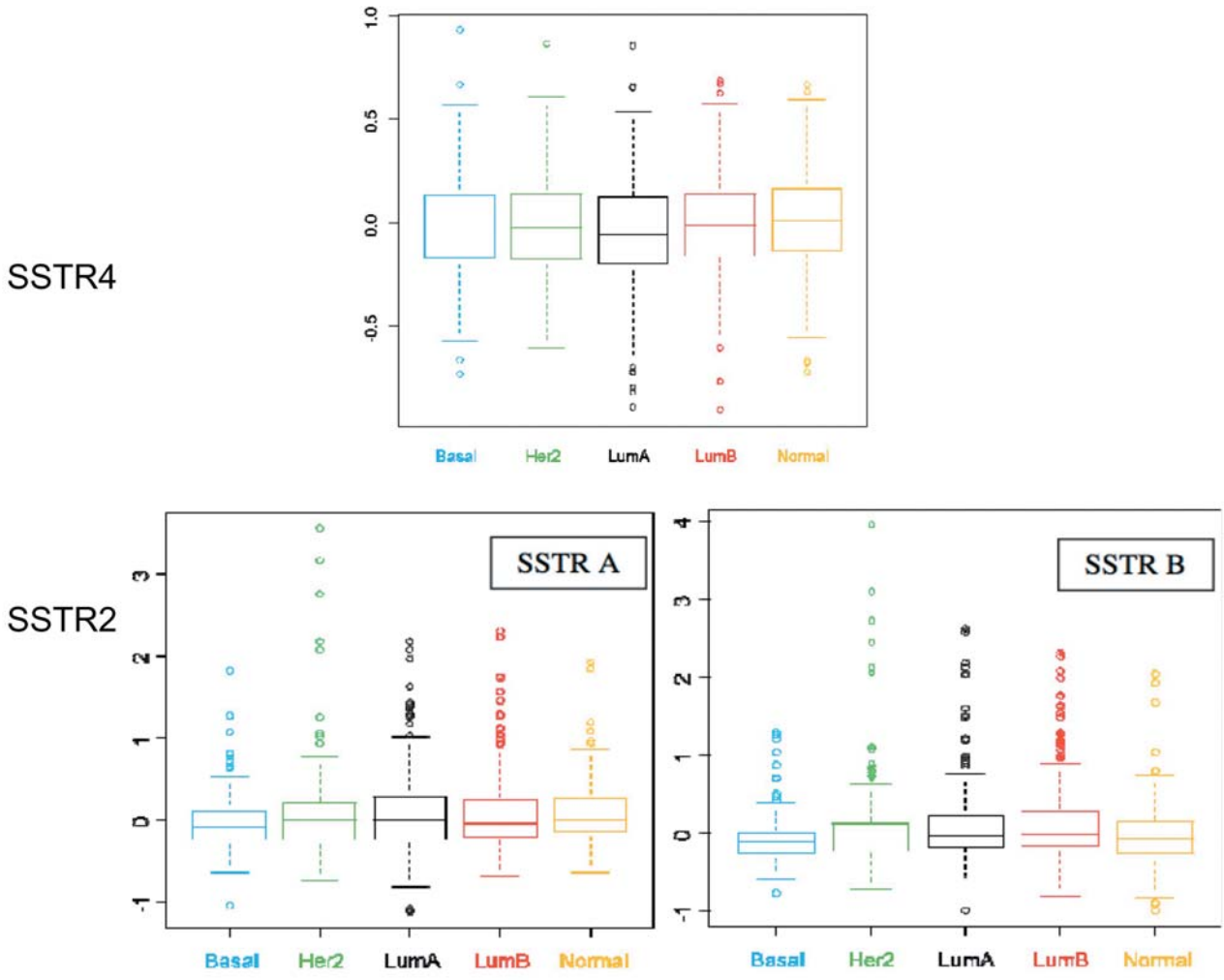

Figure 3. *Levels of SSTR2 and SSTR4 expression on molecular subtypes.*

dewaxed in xylene and rehydrated in ethanol. The antigen retrieval procedure performed prior to primary antibody incubation consisted on microwave treatment in 0.01M citric acid buffer (Merck®) (pH 6.0) twice. To quench endogenous peroxidase activity, sections were incubated in methanol containing 1.5% H_2O_2 for 30 min at room temperature. The non-specific binding was blocked by preincubation with 3% normal goat serum for 30 min at room temperature. The working dilution of antibodies was 1:100 for SSTR2 and 1:400 for SSTR4. Following overnight incubation at 4˚C in a humidified chamber with primary antibodies, the tissues were treated with anti-rabbit IgG biotinylated goat antibody and streptavidin complex (Vector Laboratories Inc. CA). The immunoreaction was visualized with 3.3'- diaminobenzidine (DAB, DAKO, Denmark) solution.

Scoring of immunereactivity. The light microscopy evaluation of the immunohistochemical staining was performed by a referent anatomopathologist. Tumor was classified by the percentage of positives cells and the intensity of staining (+++: strong staining, ++: moderate staining, +: weak staining).

Statistical analysis. We evaluated correlation between SSTR2 and SSTR4 expression and 18 tumor cells markers (ER, PR, CK18, prolactin receptor, EGFR, CK 5/6, CK14, P- cadherin, C-kit, vimentine, her2, p53, Cox2). We also realized a principal component analysis to summarize all data into a two-dimensional graphic. All statistical analyses were performed using the R package with the package FactoMineR (http://lib.stat.cmu.edu/R/CRAN/).

Gene expression analysis. To validate our results on an independent population, we also evaluated *SSTR* mRNA expression on a breast cancer data set recently used and published by Reyal *et al.* (22). The expression of SSTR2 and SSTR4 were analyzed on each molecular subtype.

The *PAM50* gene set was used for gene expression-based subtyping. The subtype classification was assigned based on the nearest of the five centroids. Because of its reproducibility in subtype classification, the final algorithm consisted of centroids constructed as described for the PAM algorithm and distances calculated using Spearman's rank correlation (23). Principal component analyses using ER, PR, HER2 and SSTR2 and SSTR4 were realized.

Results

Immunohistochemisty. 268 tumors on both TMA were analyzed. SSTR2 and SSTR4 immunostaining was an homogenous transmembrane staining of variable intensity (quoted from 1 to 3) with a proportion of marked cells quoted from 0 to 100 (Figure 1). The SSTR2 immunostaining represented 21% (47/225) of all tumors whereas SSTR4 represented 76% (166/218). We never highlighted immunostaining on healthy tissues.

Correlation with luminal tumor markers (ER, PR, CK18, prolactin receptor). The tumor overexpression of ER was significantly correlated to both SSTR2 expressions (p=0.05) with a coefficient of correlation of 0.15 and SSTR4 expression (p=0.04) with the same coefficient of correlation (0.15). The overexpression of PR was also significantly correlated to SSTR2 (p=0.001) and SSTR4 (0.04) with a coefficient of correlation of 0.30 and 0.15 respectively. The expression of CK18 and prolactin receptor was not significantly correlated with SSTR2 (p=0.73 and p=0.93 respectively). The expression of SSTR4 is significantly correlated with prolactin receptor (p=0.05) but not with CK18 (p=0.18) (Table I).

Correlation with basal tumor markers (EGFR, CK 5/6, CK14, P-cadherin, C-kit, vimentine). The expression of SSTR2 and SSTR4 was not significantly correlated with any of those markers (Table I).

Correlation with HER2 tumors markers (her2, p53, Cox2). The expression of SSTR2 and SSTR4 was not significantly correlated with any of those markers (Table I).

Principal component analysis. Principal component analysis (PCA) graphically represents vectors of various markers used to characterize breast tumors. We could individualize 3 distinct groups of markers for "luminal", "basal" and "her2" tumors. The SSTR2 subtype was correlated with luminal markers especially CK18. The SSTR4 subtype was on the opposite direction of HER2 positive tumors and also characterized luminal tumors (Figure 2).

Multi-gene analysis. From 6 breast cancer data sets (HGU-133A Affymetrix© Santa Clara, California, USA), 1,143 microarrays were selected and 1,127 were suitable for analysis. There were two probe sets targeting SSTR2 (217455_s_at, 214597_at, named a and b for analyses) and one targeting sstr4 (214556_at).

Expression of SSTR2 and SSTR4 on the different molecular subtypes were stable (Figure 3). The principal component analysis showed that SSTR2 and SSTR4 markers are independent from HER2 and correlated with luminal tumors (Figure 4).

Discussion

Immunohistochemistry of our TMA demonstrated that expression of SSTR2 and SSTR4 was significantly correlated with ER and PR and is considered to be a marker for luminal tumors. SSTR was never expressed in healthy tissues. Those results were confirmed by the multi-gene analysis.

We have demonstrated that there is an expression of SSTR2 and 4 in breast cancer TMA. However, our somatostatin receptors' expression percentage differs from those reported in the literature. We found an expression rate of 21% for sstr2 and 76% for SSTR4 while it is often reported that SSTR2 is predominant on breast cancer cells (13). This difference could be explained by the TMA patients' specifications including young patients with an increased frequency of RE negative or HER2-positive tumors.

Our analyses showed positive correlation between estrogen, progesterone receptors and SSTR2. Correlations' analysis and principal component analysis have shown that SSTR's expression is associated with luminal tumors and thought tumors overexpressing SSTR2 are mainly luminal tumors. Our results confirm the literature's basic knowledge. Indeed, Orlando *et al*. (16) showed that concentrations of *SSTR2* mRNA were significantly higher in estrogen receptor positive tumors. Similarly, *SSTR2* mRNA expression was significantly higher in "low-proliferating" breast cancers. As we didn't find SSTR2 overexpression in normal breast tissue, Orlando *et al*. also showed a significant diminution of *SSTR2* mRNA expression in normal breast tissue. Pilichowska *et al*. (15) on the other hand, found a positive SSTR2 immunostaining in breast cancer as in normal breast tissue but they studied few samples of normal breast tissue employing a non-commercialized antibody used in rats but not in humans.

In correlation with our results, it seems that overexpression of SSTR2 in breast tissue is closely-correlated with the carcinogenesis of breast cells as SSTR2 are not found in normal breast tissue but appears to be correlated with luminal breast cancers. It seems interesting to evaluate the current use of SSTR2 immunostaining to better characterize breast tumors' groups.

Also, the estrogen positive effect on SSTR2 expression's regulation in breast cancer cells (MCF7) was initially described by Xu *et al*. (24). Many other studies confirmed those results (13-15). Due to recent discovery of an additional 5' non-coding region's exon on the SSTR2 gene, Kimura *et al*. (25) analyzed the molecular pathway for *SSTR2* gene transcription's regulation. They could identify transcriptional regulatory elements in the promoter region of the *SSTR2* gene. These transcriptional regulatory elements contain estrogen's binding sites. The transcriptional activation of *SSTR2* gene is linked to two distinct promoter regions with estrogen's binding sites and they positively regulate SSTR2 transcription. These regulatory domains have

Table I. *SSTR2 and SSTR4 correlation table, (coefficient of correlation, p).*

	re	rp	ki	bcl2	egfr	ck5	ck14	her2k	ck18	ecadh	p53	ckit	p63	vim	cox2	prolacr	muc1	sstr2	sstr4
re	NA	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,50	0,00	0,00	0,03	0,00	0,86	0,00	0,33	0,10	0,04
rp	0,47	NA	0,00	0,00	0,00	0,00	0,01	0,01	0,00	0,07	0,00	0,26	0,41	0,00	0,07	0,44	0,04	0,00	0,04
ki	-0,37	-0,26	NA	0,00	0,00	0,01	0,15	0,09	0,00	0,13	0,00	0,00	0,00	0,00	0,62	0,86	0,05	0,15	0,31
bcl2	0,55	0,29	-0,24	NA	0,00	0,00	0,08	0,00	0,00	0,00	0,00	0,58	0,24	0,00	0,87	0,00	0,94	0,99	0,00
egfr	-0,49	-0,32	0,28	-0,30	NA	0,10	0,00	0,35	0,00	0,25	0,16	0,00	0,08	0,00	0,97	0,00	0,72	0,19	0,38
ck5	-0,34	-0,24	0,17	-0,22	0,11	NA	0,00	0,52	0,02	0,20	0,01	0,00	0,72	0,00	0,27	0,02	0,08	0,21	0,71
ck14	-0,23	-0,18	0,10	-0,12	0,45	0,19	NA	0,48	0,00	0,39	0,60	0,01	0,93	0,00	0,16	0,14	0,15	0,37	0,39
her2k	-0,29	-0,17	0,11	-0,38	0,06	0,04	-0,05	NA	0,80	0,99	0,00	0,27	0,57	0,76	0,00	0,08	0,08	0,50	0,22
ck18	0,31	0,34	-0,25	0,24	-0,34	-0,16	-0,21	0,02	NA	0,82	0,06	0,13	0,05	0,00	0,22	0,00	0,04	0,94	0,18
ecadh	-0,05	-0,12	0,10	0,21	0,08	0,09	0,06	0,00	-0,02	NA	0,27	0,00	0,75	0,25	0,01	0,10	0,27	0,10	0,38
p53	-0,36	-0,23	0,20	-0,31	0,09	0,19	-0,04	0,31	-0,13	0,08	NA	0,05	0,01	0,20	0,00	0,95	0,85	0,32	0,95
ckit	-0,26	-0,08	0,22	-0,04	0,24	0,35	0,18	0,07	-0,10	0,34	0,13	NA	0,17	0,00	0,68	0,02	0,43	0,07	0,12
p63	-0,14	-0,06	0,20	-0,08	0,12	-0,02	0,01	0,04	-0,13	-0,02	0,17	0,09	NA	0,00	0,23	0,53	0,38	0,24	0,71
vim	-0,45	-0,31	0,30	-0,28	0,33	0,26	0,37	-0,02	-0,39	-0,08	0,09	0,23	0,27	NA	0,34	0,00	0,15	0,30	0,72
cox2	-0,01	-0,12	0,03	-0,01	0,00	-0,08	0,10	0,19	-0,08	0,18	0,20	0,03	0,08	-0,07	NA	0,08	0,18	0,28	0,66
prolacr	0,27	0,05	0,01	0,32	-0,21	-0,16	-0,10	0,12	0,23	0,11	0,00	-0,16	-0,04	-0,26	0,12	NA	0,26	0,79	0,05
muc1	0,09	0,18	-0,18	0,01	-0,03	-0,16	-0,13	0,16	0,19	-0,10	0,02	-0,07	-0,08	-0,13	-0,12	0,10	NA	0,85	0,37
sstr2	0,12	0,25	0,10	0,00	-0,09	-0,09	-0,06	-0,05	0,01	-0,12	-0,07	-0,13	0,08	-0,07	0,08	-0,02	-0,02	NA	0,45
sstr4	0,15	0,15	0,07	0,32	-0,06	-0,03	0,06	-0,09	0,10	0,06	0,00	0,11	0,03	-0,03	0,03	0,14	-0,09	0,05	NA

been found in T47D breast cancer cells that therefore overexpress estrogen receptor but are not found in cervical cancer. It seems that estrogens positively regulate the transcription of SSTR2 with these two distinct regions and that they are only expressed on hormone-sensitive tumors.

With no SSTR2 in healthy breast tissue, its correlation with luminal tumors and its regulation by estrogens, it can be hypothesized that SSTR2 expression in breast tumors is a "cellular repair" pathway induced by tumor cells.

The use of somatostatin analogs in breast cancer remains controversial and results available to date remain conflicting with regard to its impact on survival. *In vitro*, several studies have demonstrated somatostatin analogs' anti-proliferative action on breast cancer cells (26-28). Indeed, a group has shown that SSTR2 overexpression plays an anti-proliferative part in the estrogen-dependent MCF-7 cells by inducing apoptosis and decreasing EGFR expression (27). Using a somatostatin analogue associated with doxorubicin, another group has demonstrated an inhibition of tumor cell growth on breast cancer cells without estrogen receptors (MDA-MB-231) (26). A third team has reported a significant inhibition of growth on MDA-MB-231, MX- 1 and MCF-7 cells using a combination of somatostatin analogs (AN-238) and doxorubicin. In animal models, it induces inhibition of tumor growth for tumors overexpressing SSTR2 and 5 xenografted to nude mice (28). Both "*in vitro*" data and animal models suggest that use of somatostatin's analogs could induce positive regulation of SSTR expression and thought to block tumor progression.

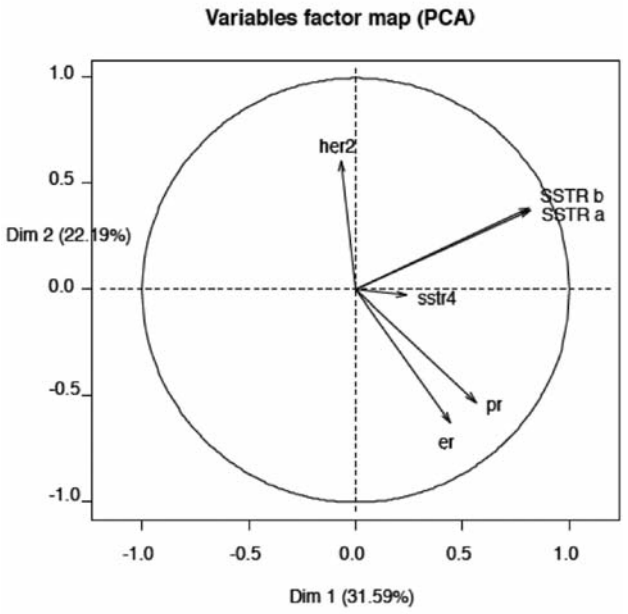

Figure 4. *Principal component analysis on independent breast cancer data set.*

In metastatic breast cancer, a randomized study comparing tamoxifen combined with octreotide *versus* tamoxifen-plus-placebo on first-line treatment for metastatic patients didn't show any progression-free survival differences between the

two groups (29). Another study involving 22 patients with metastatic breast cancer showed improvement of progression-free survival by the addition of somatostatin analogs and an anti-prolactin molecule to tamoxifen (84 weeks vs 33 weeks) (30). A literature review including 210 patients treated for metastatic breast cancer with somatostatin analogs showed better tumor response rate especially in first-line metastatic (69.5 % *versus* 28.5 %, p=0.006) and in patients with at least two metastases (31).

In the adjuvant setting, using the combination tamoxifen - octerotide *versus* tamoxifen-alone in 667 patients, Pritchard *et al*. did not find any differences in terms of disease-free survival and overall survival between the two groups (32). The discrepancy of these results could be explained by the lack of patients' selection. It, therefore, seems interesting to specifically analyze this signaling pathway to permit patient selection and potential specific treatment. We believe that molecular classes study for breast cancer can help refine sub-tumor groups and that somatostatin receptors may eventually find their place in clinic as hormone receptor or her2.

In conclusion, it appears that somatostatin receptor expression is a marker for luminal breast cancer related to tumor differentiation. When examining the "*in vitro*" studies' results, it seems interesting to assess somatostatin analogues' antitumor effects on luminal breast cancers.

Acknowledgements

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