Expression and Localization of Serotonin Receptors in Human Breast Cancer

PRADEEP KUMAR KOPPARAPU[1], MARTINA TINZL[2], LOLA ANAGNOSTAKI[3], JENNY LIAO PERSSON[1] and NISHTMAN DIZEYI[2]

[1]*Division of Experimental Cancer Research, Department of Laboratory Medicine,*
[2]*Division of Urological Research, Clinical Research Center,*
[3]*Department of Clinical Pathology, Lund University, Malmö, Sweden*

Abstract. *The aim of this study was to examine the expression of serotonin receptors in patients with breast cancer and to explore their utility as diagnostic and prognostic markers. Immunohistochemical analysis was performed to examine the expression of serotonin (5-HT) receptor subtypes 1A, 1B, 2B and 4 in a tissue microarray containing tumor specimens from 102 patients. Statistical analysis was performed to correlate the expression of these proteins with regard to clinical parameters. We found that all four serotonin receptors (5-HTRs) exhibited different expression patterns in breast cancer specimens. In general strong staining for 5-HTR1A was observed on the membrane of cancer cells but it was detected only in the cytoplasm of non-malignant cells. 5-HTR1B and 5-HTR2B were predominantly expressed in the cytoplasm of breast cancer cells, while 5-HTR4 was exclusively found in the nucleus of malignant and non-malignant cells. Correlation analysis revealed a significant correlation of 5-HTR2B with estrogen receptor-α (ER-α) and 5-HTR4 with ER-α and progesterone (PR). In conclusion, the different expression patterns and subcellular localization of 5-HTRs in breast cancer may reflect their role in breast cancer progression.*

Breast cancer is one of the diseases for which treatment regimens are based on the profile of prognostic markers in addition to conventional histological grading. Steroid receptors (1), proto-oncogenes such as Human Epidermal Growth Factor Receptor-2 (HER-2) (2, 3) and mutations in the *p53* gene (4) have all been correlated to poor prognosis. However, the predictive value of these prognostic markers each alone and in combination, has been of limited benefit so far. Therefore, additional parameters may provide with better treatment options for patients with breast cancer. In this report we have extended our earlier studies (5-7) to analyze serotonin receptors in cancer of the human breast, another hormonally-regulated organ which is prone to microenvironmental changes.

Serotonin (5-HT), a well-known neurotransmitter, which also functions as a hormone in systems outside of the central nervous system. The functional diversity of 5-HT is reflected by the variety of pharmacological effects mediated by the seven different families of 5-HT receptors (5-HTR1 to 7). All except 5-HTR3 are G-protein-coupled receptors (GPCR), including G_i (5-HTR1), G_s (5-HTR4, 6 and 7) and $G_{q/11}$ (5-HTR2 and 5) (8). The expression of 5-HTRs has been demonstrated in patient tissues such as breast cancer, in lymphomas derived from germinal center B-cells (9, 10), prostate and ovarian cancer (5, 7), and in hepatocellular cancer (11, 12). Out of these, isoforms of receptor subtypes-1 and -2 have been reported to exert the mitogenic effect of 5-HT in breast cancer cells (13), prostate cancer cells (5, 14) hepatocellular carcinoma (12), human placental choriocarcinoma cells (15) and lung cancer (16). Collectively, these data indicate that 5-HT promotes cell growth and survival *via* activation of these receptor subtypes. As yet, the exact mechanism of the mitogenic action of 5-HT in the context of cancer has not been fully-elucidated, however, studies have shown that receptor subtypes 5-HTR1B and 5-HTR2A can directly activate the extracellular signal-regulated kinase (ERK) pathway and c-jun *N*-terminal kinase (JNK) (17, 18). Among the 5-HT receptors, 5-HTR2B has been described to mediate proliferation *via* cross-talk with receptor tyrosine kinase (RTK) (19). The same authors also showed that 5-HTR2B contributes to tumor formation in nude mice. Receptor subtype on the other hand, is mainly related to hormone secretion and its role in tumor regulation is yet to be identified. In two separate studies, agonists to 5-HTR4 were shown to stimulate cortisol secretion in patients with adrenal tumor, or bilateral adrenal hyperplasia and Cushing's

Correspondence to: Nishtman Dizeyi, Ph.D., Division of Urological Research and Department of Laboratory Medicine, Lund University, Clinical Research Center, 205 02 Malmö, Sweden. Tel: +4640391102, Fax: +4640391222, e-mail: nishtman.dizeyi@med.lu.se

Key Words: 5-HTRs, serotonin, malignant, non-malignant, invasive ductal carcinoma, invasive lobular carcinoma, breast cancer.

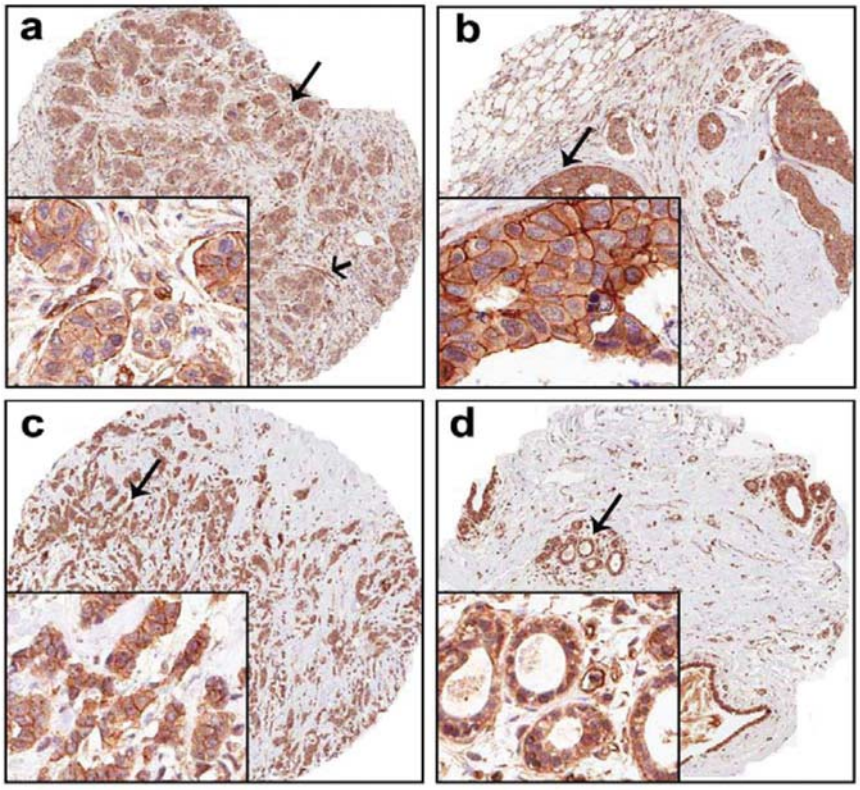

Figure 1. *Expression of serotonin receptor-1A (5-HT1A), as estimated by immunohistochemistry using a specific antibody targeting 5-HTR1A. In invasive ductal carcinoma (a), ductal carcinoma in situ (b), lobular carcinoma (c) and non-malignant specimen (d). Core magnification: ×6 and inset ×20. Expression of 5-HTR1A in blood vessel tissues is indicated by a broad arrow.*

syndrome (20, 21). On this respect, 5-HTR4 may contribute to tumor progression by stimulating secretion of hormones and other growth factors.

The results from recent studies strongly support the important role of 5-HT in the regulation of the mammary gland, including normal tissue and cancer. For instance, Pai and colleagues have shown that alteration of 5-HT in mammary epithelial cells led to dysregulation of epithelial homeostatic systems (9, 22), a condition which may be involved in the tumorigenesis of breast cancer. Furthermore, Rybaczyk *et al*. addressed a central but overlooked issue in women´s health, namely the impact of estrogen and serotonin interrelation in tumorigenesis of breast cancer (23). In another study, Hernandez and co-workers showed that 5-HT induced parathyroid hormone-related protein (PTHrP) and the metastasis-associated transcription factor RUNX2/CBFA1 in human breast cancer cells (24). They concluded that the autocrine 5-HT-PTHrP signaling system is essential for induction of bone-regulating factors in the normal mammary gland and in breast cancer cells. To gain insight into the possible association between the presence of 5-HTRs and breast cancer progression in actual human breast cancer tumors, we sought to examine the expression of serotonin receptors in a large cohort of patients with breast cancer. The presence of 5-HTRs in cancer cells may allow us to consider one or more of these receptors as prognostic markers.

Materials and Methods

Tissue specimens and tissue microarrays. The tissue microarray (TMA) used in this study consisted of primary breast cancer specimens purchased from pantomics (http://www.pantomics.com/; Pantomics, Inc., Richmond, CA, USA). TMA tissue cores were identified as non-malignant lesions (5/102), ductal carcinoma *in situ* (DCIS), (6/102), ductal carcinoma (85/102), and invasive lobular cancer (6/102) by histopathological investigations. Stained slides were examined by ND and LA. The study was approved by the Ethics committee of the Lund University and the Helsinki Declaration of Human Rights was strictly observed.

Antibodies and immunohistochemistry. The staining was estimated by staining intensity as well as by the percentage of cells stained. Immunohistochemistry was conducted on 5-μm-thick TMA tissue sections, as described elsewhere (5). Antibodies used were against 5-HTR1A (diluted 1:300), 5-HTR1B (diluted 1:400) and 5-HTR2B

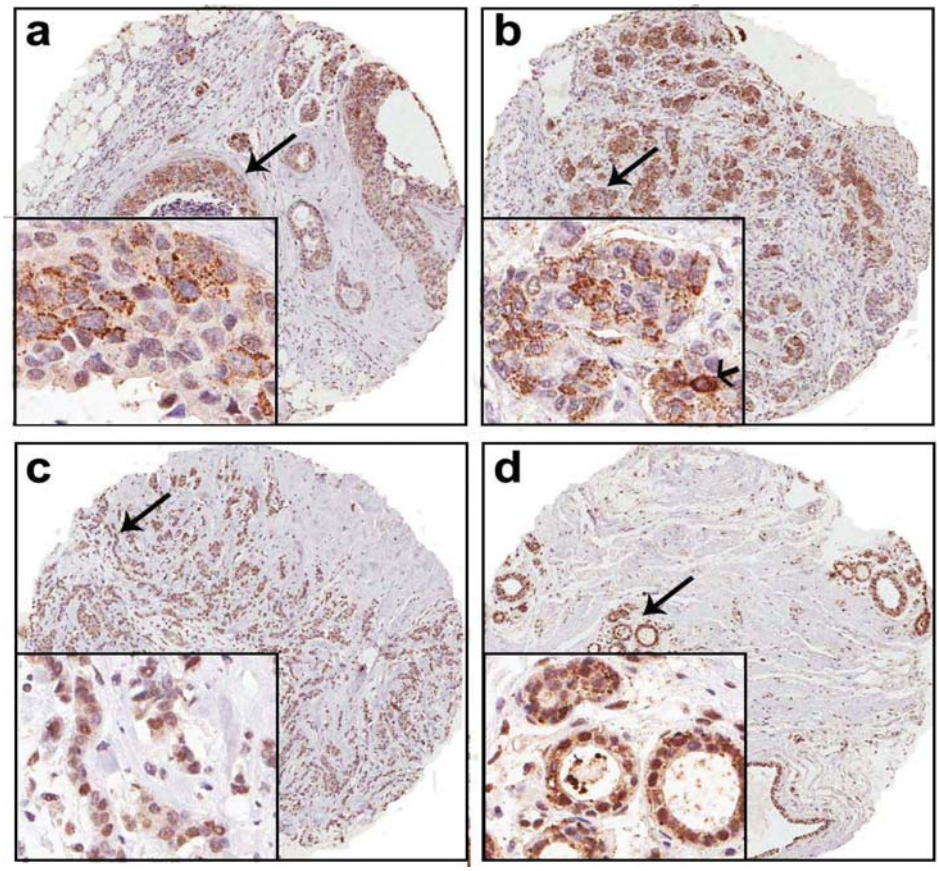

Figure 2. *Expression of serotonin receptor-1B (5-HT1B) as estimated by immunohistochemistry using specific a antibody targeting 5-HTR1B. In ductal carcinoma in situ (a), invasive ductal carcinoma (b), lobular carcinoma (c) and non-malignant specimen (d). Core magnification: ×6 and inset ×20. Expression of 5-HTR1B in neuroendocrine-like cells is indicated by a broad arrow.*

(diluted 1:400), purchased from Santa Cruz (Santa Cruz Biotechnology, CA, USA), and against 5-HTR4 (1:000) purchased from Acris (Acris antibodies, Inc, San Diego, CA, USA). Antibodies against 5-HTR1A and 5-HTR4 were raised in rabbit and 5-HTR1B and HTR2B were raised in goat.

Interpretation of immunohistochemical staining. Expression levels were scored on a scale of 0 to 3, based on staining intensity and the proportion of positive tumor cells, by an expert pathologist (LA) and a scientist (ND). The staining was scored as 0 if no cancer cells were stained, 1 if staining was weakly-positive, 2 if staining was moderately-positive, or 3 if staining was strongly positive. The sections were viewed with an Olympus A×70 microscope at a magnification of ×20. The slides were scanned and microphotographs were taken by using a scanner (ScanscopeCS; Aperio, Vista, CA, USA).

Statistical methods. Possible pair-wise correlations between groups were analyzed using the Spearman rank-correlation test. All statistical tests were two-sided and *p*-values less than 0.05 were considered to be statistically significant. Statistical analysis was carried out using the SPSS 20 (SPSS Inc., Chicago, IL, USA).

Results

The general characteristics of the 102 patients included in immunohistochemical analysis are summerized in Table I. The expression of 5-HTR1A, -1B, -2B and 5-HTR4 in breast cancer specimens on the TMA are summerized in Table II. In Table III, the correlation coefficients between levels of expression of 5-HTR1A, 5-HTR1B, 5-HTR2B, 5-HTR4 with ER-α, PR, HER-2 and with tumor grade in tissue specimens from patients with breast cancer-only (N=97) was determined using Spearman's test. The expression pattern of each protein assessed by immunohistochemical analysis is shown in Figures 1 to 4.

Serotonin receptor subtype-1A expression. Overall immunoreaction of 5-HTR1A receptor protein was demonstrated in all 102 samples analyzed. The expression of 5-HTR1A was variable, from moderate to strong, regardless of tumor type and stage (Tables I and II). The majority of ductal carcinoma specimens, including both invasive and in DCIS,

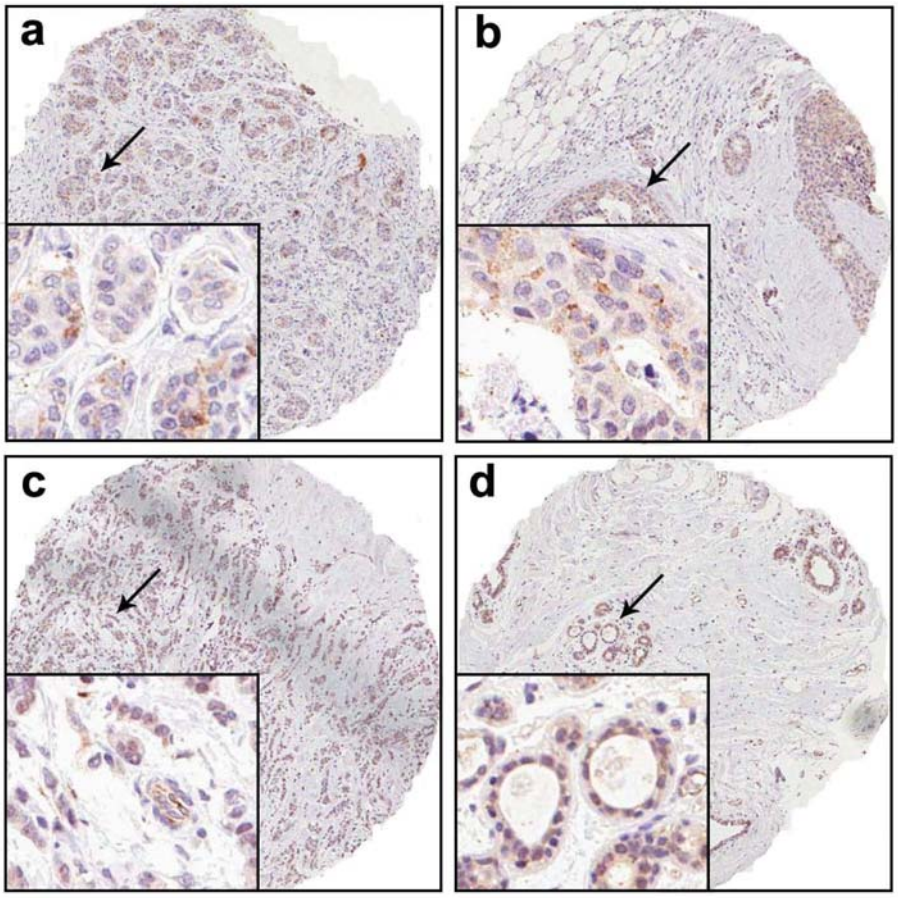

Figure 3. *Expression of serotonin receptor-2B (5-HT2B) as estimated by immunohistochemistry using a specific antibody targeting 5-HTR2B. Invasive ductal carcinoma (a), ductal carcinoma in situ (b), lobular carcinoma (c) and non-malignant specimen (d). Core magnification: ×6 and inset ×20.*

Table I. *General characteristics of 102 patients included in the immunohistochemical analysis.*

Clinical properties	No. of patients (%)
Total	102
Age (years)	
Median	46
Range	18-72
Pathlogical grade	
Non-malignant	5 (4.9)
0	6 (5.9)
1	18 (17.6)
2	55 (53.9)
3	16 (15.6)
Unknown	2 (2.0)
Primary tumor stage (97/102)	
pTis	6 (6.19)
pT2	44 (45.36)
pT3	34 (35.05)
pT4	13 (13.40)
Lymph node status	
pN0	52 (53.61)
pN+	45 (45.36)

displayed high expression of 5-HTR1A in the plasma membranes (Figure 1a and b). In contrast, in lobular carcinoma, we observed strong 5-HTR1A staining predominantly in the cytoplasm, and also in the cell membrane (Figure 1b). In summary, the plasma membrane was the major sub-cellular compartment where expression of this receptor was detected. In contrast to what was observed in breast cancer cells, 5-HTR1A expression was detected in the cytoplasm of non-malignant cells (Figure 1c). Moderate and strong levels of 5-HTR1A were also observed in blood vessels (Figure 1a, broad arrow).

Serotonin receptor subtype-1B expression. In contrast to 5-HTRA1, the expression of 5-HTR1B was less pronounced and highly variable in breast cancer specimens. A total of 35 out of 97 breast cancer specimens lacked detectable expression of 5-HTR1B, 23 out of 97 had a moderate level, while 6 out of 97 had a high level of receptor expression (Table I and II). 5-HTR1B staining was mainly located in the cytoplasm of tumor cells (Figure 2a) but in a few cases (n=7, figure not shown) nuclear staining of 5-HTR1B was also

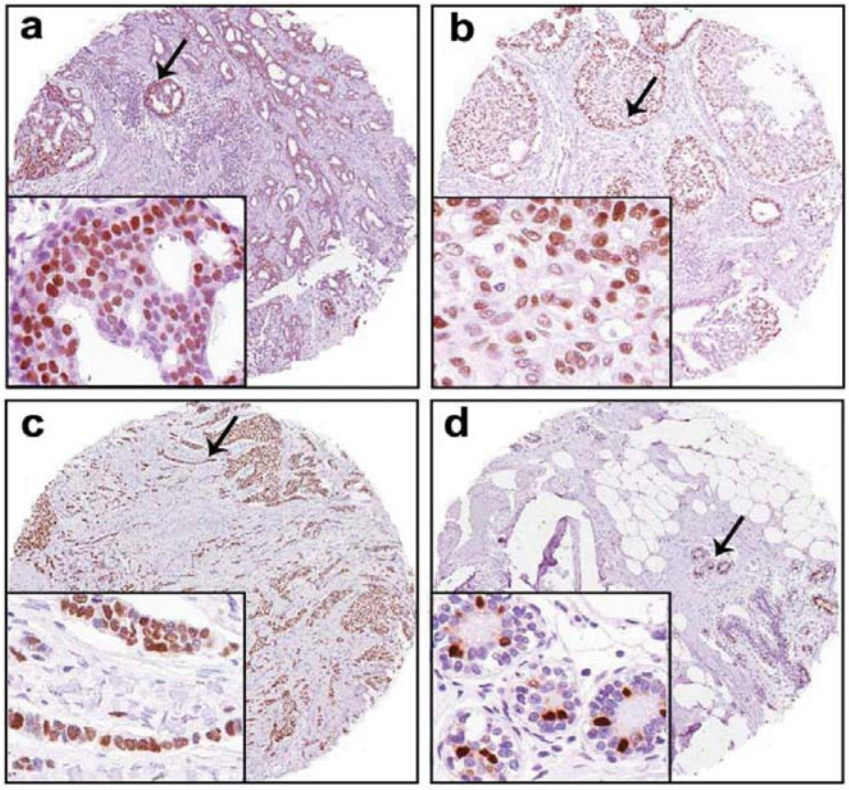

Figure 4. *Expression of serotonin receptor-4 (5-HT4), as estimated by immunohistochemistry using a specific antibody targeting 5-HTR4. Invasive ductal carcinoma (a), ductal carcinoma in situ (b), lobular carcinoma (c) and non-malignant specimen (d). Core magnification: ×6 and inset ×20.*

Table II. *Evaluation of serotonin receptor subtypes -1A, -1B, -2B and -4 in tissue microarray, including malignant and non-malignant specimens.*

	5-HTR1A	5-HTR1B	5-HTR2B	5-HTR4
Group (score)	N (%)	N (%)	N (%)	N (%)
Low staining (1)	-----	37 (36.3)	45 (44.1)	1 (0.9)
Moderate staining (2)	32 (31.4)	23 (22.5)	18 (17.6)	19 (18.6)
Strong staining (3)	70 (68.6)	6 (5.9)	5 (4.9)	22 (21.6)
No staining (0)	-----	36 (35.3)	34 (33.3)	60 (58.9)
Total	102	102	102	102

Table III. *Correlation coefficients between levels of expression of serotonin receptors (5-HTRs) with estrogen receptor (ER), progesterone receptor (PR), Ki67, Human Epidermal Growth Factor Receptor-2 (HER-2) and with tumor grade in tissue specimens from patients with breast cancer (N=97) was determined using Spearman's test.*

Index	ER	HER-2	Ki-67	PR	Tumor grade
5-HTR1A	–0.48	0.144	0.060	0.078	–0.089
5-HTR1B	–0.072	0.182	–0.159	–0.190	–0.002
5-HTR2B	0.278**	0.182	0.017	0.141	–0.095
5-HTR4	0.603**	–0.074	–0.005	0.831**	0.021

Two-tailed test was applied to evaluate the level of significance. ***p*=0.01.

observed in tumor cells. 5-HTR1B expression was observed in DCIS, being higher at the margins compared to the core of the tumor (Figure 2b). The majority of samples displayed a weak to moderate level of 5-HTR1B expression. A high level of 5-HTR1B was observed in the cytoplasm of invasive lobular cancer (5/6) (Figure 2c). Furthermore, in two tumor samples identified as invasive ductal carcinoma, diffuse and strong cytoplasmic staining of 5-HTR1B was found in few cells possessing neuroendocrine (NE)-like features (Figure 2a, broad arrow). Additionally, an overall weak expression of 5-HTR1B was demonstrated in the cytoplasm of non-malignant epithelial cells (5/5) (Figure 2d). Blood vessel cells were weakly stained for 5-HT1B.

Serotonin receptor subtype-2B expression. The expression pattern of 5-HTR2B was similar to that of 5-HTR1B in terms of heterogeneous staining and cellular localization (Table I and II). A total of 32 out of 97 breast cancer specimens lacked 5-

HTR2B expression and 45 out of 97 exhibited weak staining. A moderate level of 5-HTR2B was observed in invasive ductal carcinoma and DCIS (15/97) (Figure 3a and b). Only four breast cancer specimens exhibited strong 5-HTR2B cytoplasmic staining in tumor cells. In five tumor samples identified as invasive ductal carcinoma, contrasted and diffuse strong cytoplasmic staining was detected in cells possessing NE-like features (Figure 3a, broad arrow). There was no difference in the staining intensity or subcellular localization between ductal and lobular carcinomas (Figure 3c). In non-malignant samples, moderate (2/5) and strong (3/5) staining was observed. Similarly to 5-HTR1A, the 5-HTR2B immunoreaction was seen in cells related to blood vessels.

Serotonin receptor subtype-4 expression. In contrast to the aforementioned receptors, the expression of 5-HTR4 was found exclusively in the nucleus. We observed strong to moderate expression of 5-HTR4 in the nucleus of tumor cells and non-malignant cells (Tables I and II). Interestingly, 5-HTR4 expression was absent from more than 60% of malignant samples (60/97). A total of 37% specimens exhibited a moderate to high level of 5-HTR4 expression regardless of tumor type (Figure 4a-c). A high level of 5-HTR4 expression was observed in 17 out of 97 (17.5%) of cancer specimens, whereas moderate staining of 5-HTR4 was detected in 19 out of 97 (19.6%) cancer specimens. The invasive ductal carcinomas exhibited higher level of 5-HTR4 expression than that in the lobular carcinomas (Figure 4a and b). Interestingly, 5-HTR4 expression was mainly found in the patients aged less than 50 years compared to those older than than 50 years, however, we were not able to show statistically significant positive correlation due to the small number of patients. All non-malignant specimens (5/5) stained positive for 5-HTR4 but in a few cells (Figure 4d). No positive staining for 5-HTR4 was seen in blood vessels.

Furthermore, to address the question of *5-HTR* mRNA expression signatures in breast cancer, we examined the mRNA expression of 5-HTRs using the *Oncomine* database (http://www.oncomine.org). Among the receptor isoforms, 5-HTR1B, -1D, -1E, -1F, -2A, -2B, -2C, -3, -4, -5A and -7 mRNA expression were found in the MCF-7 breast cancer cell line and in non-malignant tissues and malignant breast tumor tissues, as previously described (9).

Correlation analysis. Having identified the expression and localization of 5-HTRs, we next evaluated whether there are correlations between 5-HTRs and known breast cancer biomarkers including ER-α, PR, Ki67, HER-2 and tumor grade (0 to 3 of which 0 is DCIS, grade 1 non-invasive intraductal and 2 and 3 are invasive cancers). We observed that 5-HTR2B was significantly correlated with ER-α (p=0.008), whereas 5-HTR4 significantly correlated with ER-α (p<0.001) and PR (p<0.001) (Table III). Nevertheless, there was no correlation between any of the receptor subtypes with tumor grade (Table III).

Discussion

In the present study, we have shown that all four 5-HTR subtypes examined were differentially expressed in human breast cancer. The diversity of the expression pattern of each receptor subtype may be a useful tool for understanding the distribution and function of each subtype in the pathogenesis of breast cancer. We observed strong immunoreaction for 5-HTR1A on the plasma membrane of ductal carcinoma including invasive and non-invasive forms. Lobular carcinoma exhibited strong immunoreaction in both the cytoplasm and cell membrane, and in non-malignant cells, strong immunostaining was mainly present in the cytoplasm. The expression of receptor subtypes 5-HTR1B and 5-HTR2B was less pronounced and was predominantly found in the cytoplasm. The intensity of immunoreactivity was moderate in ductal but strong in lobular carcinomas. The most striking result found in this study was the presence of 5-HTR4, which was exclusively nuclear.

The data presented here were consistent with our previously reported results (5, 7), in which we showed strong expression of 5-HTR1A in advanced prostate cancer, a subset of ovarian cancer, and in non-malignant cells. Subcellular localization of 5-HTR1A seems to be tissue-dependent, being cytoplasmic in benign and membranous in malignant cells in a pattern similar to that for HER-2. For decades, HER-2 has been used as a prognostic marker of breast cancer. High levels of either *HER-2* gene amplification or protein expression tends to result in a poorer breast cancer prognosis (25). Accordingly, 5-HTR1A may be used as a prognostic marker for treatment outcome. Strong expression of 5-HTR1B and 2B in a selective number of tissue cores, but low or lack of expression in non-malignant cells, indicate the possible role of these two receptors in tumor progression. This result was consistent with the expression of 5-HTR1B and -2B in hepatocellular carcinoma (12), where a statistically significant higher level of the mentioned receptors but not of 5-HTR1A was found in cancer, compared to normal tissue. 5-HTR2B has been implicated in cancer progression in several organs (26, 27). Launay *et al*. showed that 5-HT induced tumor transformation in a mouse fibroblast cell line stably transfected with 5-HTR2B, and that this receptor subtype is necessary for the induction of tumor formation in nude mice. Thus, 5-HTR2B may play a key role in the early stages of carcinogenesis. These data suggest that the serotonin receptor function in cancer cell regulation is context-dependent.

Importantly, similarly to previous studies 5-HTR2B and 5-HTR1A were also expressed in blood vessel-related cells

(6, 7). Although the action of 5-HT and vascular endothelial growth factor (VEGF) is through different membrane receptors, it has been shown that VEGF and 5-HT activate an identical set of signaling kinases (28), demonstrating that the downstream angiogenic signaling pathways of VEGF and 5-HT cross at several points, or partially overlap. On this respect, 5-HT released from platelets can directly interact with adjacent endothelial cells and lead to activation of angiogenic pathways, and thus, promote tumor growth through angiogenesis.

A novel finding in this study is the expression of 5-HTR4, which was exclusively nuclear. This finding contradicted our previous finding by which we observed 5-HTR4 in the cytoplasm of prostatic and ovarian cancer tissues. However, in agreement with the results obtained for the VEGF receptor (29), nuclear localization of GPCR can occur depending on the cell type and circumstances. It has been shown that the membrane receptor VEGFR2 also has a nuclear localization and can activate multiple signaling pathways upon stimulation (30). The authors have identified a new mechanism by which VEGFR2 activates its own promoter, which could be involved in amplifying the angiogenic response. Therefore, we believe that a similar scenario occurs in breast cancer cells. Accumulation of 5-HTR4 in the nucleus under certain conditions may enhance signaling pathways. Currently, there are no known mechanisms that would ensure specific 5-HTR4 localization to the nucleus and it may have as yet undiscovered nuclear functions. Furthermore, we hypothesize that the presence of 5-HTR4 as a nuclear receptor may be involved in the process of tumor progression through 5-HT-induced regulation of estrogen/serotonin in the micro-environment. Nuclear expression of 5-HTR4, together with its correlation to the age and presence of ER-α and PR makes this receptor a functionally significant protein and warrants further attention.

The localization of 5-HTRs in different compartments of breast carcinoma raises the question of what function each receptor has in the pathogenesis of this gland. Which receptor subtype is more relevant in transforming mammary gland cells from benign to malignant cells? Generally, internalization of GPCRs is initiated by agonist-mediated receptor phosphorylation by GPCR kinases (31, 32). Subsequent to internalization, each receptor subtype initiates a distinct downstream signaling cascade, such as the one of mitogen-activated protein (MAP) kinase (33). The presence of 5-HTR in the cytoplasm, observed in our previous studies along with other experimental data (5, 34) suggest that internalization is evident for the 5-HTRs. This also raises the possibility that the presence of 5-HTRs on the membrane and/or in cytoplasm may be a condition that selectively promotes or inhibits transformation towards aggressiveness and could have a significant physiological relevance in breast cancer.

In the present study, to our knowledge, we give the first comprehensive analysis on the subcellular localization of 5-HTR subtypes in human breast cancer. Our results show strong nuclear expression of 5-HTR4 in cancer cells, suggesting the importance of serotonin and its receptor in breast cancer. Although there are some limitations to the current study, including the number of enrolled patients, the results clearly indicate the significance of one or more subtypes, which may be used as prognostic or diagnostic markers. Further studies are, therefore, warranted in a larger series of patients with a larger number of controls.

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