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Histamine H4 Receptor Expression in Triple-negative Breast Cancer: An Exploratory Study

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Summary

Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype. There are neither universally accepted prognostic markers nor molecular targets related to TNBC. The histamine H4 receptor (H4R) has been characterized in TNBC experimental models, demonstrating its critical role in tumor development and progression. In this study, H4R expression was compared in breast cancer subtypes and correlated with clinical features using The Cancer Genome Atlas data (Pan-Cancer Atlas). The H4R status was further evaluated by immunohistochemistry in 30 TNBC human samples in relation to clinicopathological parameters. Results indicate that H4R was downregulated in basal-like/TNBC compared with luminal A and normal breast-like tumors. The higher expression of H4R was associated with improved progression-free and overall survival outcomes in basal-like/TNBC. H4R immunoreactivity was detected in about 70% of tumors, and its expression was positively correlated with the levels in the histologically normal peritumoral tissue. High H4R expression in peritumoral tissue correlated with reduced number of lymph node involvement and unifocal TNBC, while it was associated with increased patient survival. In conclusion, the H4R might represent a potential prognostic biomarker in TNBC. Further studies in large cohorts are needed to better understand the significance of H4R in breast cancer biology. (J Histochem Cytochem 70: 311–322, 2022)

Keywords

histamine H4 receptor, immunohistochemistry, metastasis, prognostic marker, triple-negative breast cancer

Introduction

Breast cancer is the most frequently diagnosed neoplasia and the leading cause of cancer-related mortality among women worldwide.[1,2] These tumors are heterogeneous, and present distinct histopathological patterns and clinical behavior. Different molecular subtypes of breast cancer with distinctive biological features have been identified, based on gene expression profiles of human tumors. They include luminal A, luminal B, basal-like, normal breast-like, and human epidermal growth factor receptor 2 (HER2)-positive subgroups with different incidence and prognosis.[3]

Within the pathology-based triple-negative tumors, the vast majority fall into the basal-like molecularly classified subtype (around 80%, depending on the study). Triple-negative breast cancer (TNBC) accounts

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for about 10–20% of all breast cancers, and it is considered the most aggressive subtype, lacking estrogen receptor (ER), progesterone receptor (PR), and HER2.[4–9] It is associated with poor prognostic features including higher nuclear grade, increased incidence of metastases, and a short recurrence-free interval. Furthermore, there are neither universally accepted prognostic markers to predict outcomes nor well-defined molecular targets in TNBC subtype.[3,5,6,8] Therefore, there is an urgent need to establish prognostic factors and to improve TNBC treatments, focusing on the development of novel biomarkers to identify potential patients for a personalized therapeutic approach.[5–9] In this regard, one of the most important conditions is an adequate characterization of the tumors and the understanding of the mechanisms involved in TNBC heterogeneity.

The histaminergic system is one of the most interesting and complex biological pathways involved in cancer disease. High histamine biosynthesis and content together with histamine receptors have been reported in different tumors, including gastric, colorectal, esophageal, oral, pancreatic, liver, lung, skin, blood, and breast cancers.[10,11] The histamine H4 receptor (H4R) was discovered two decades ago, and it has contributed to a better understanding of the histamine roles in health and disease, opening new perspectives in neoplastic research.[10–12] In breast cancer and particularly in TNBC, H4R expression has been well characterized in different in vitro and in vivo experimental models, demonstrating its critical role in the regulation of tumor proliferation, development, and progression. The administration of histamine or H4R agonists diminished the tumor growth in both immune-deficient and immune-competent TNBC preclinical experimental models.[12–16] The analysis of The Cancer Genome Atlas (TCGA) data showed that the H4R gene expression is impaired in primary tumors compared with normal tissue in different cancer types.[11,17] However, the immunohistochemical expression of H4R in TNBC and its prognostic value is completely unknown.

In the present exploratory work, we first compared the H4R expression in breast cancer subtypes using publicly available TCGA data, and correlated H4R mRNA expression with clinical attributes. We corroborated transcriptomic data by analyzing the H4R status in TNBC human samples in relation to clinicopathological parameters. This study will improve the knowledge of the role of H4R in breast cancer progression and could provide a venue for the development of a new diagnostic tool and/or therapeutic target, particularly for those subtypes of breast cancer with limited therapeutic options.

Materials and Methods

In Silico Data Analysis

The cBioPortal for Cancer Genomics is an open-access resource for interactive exploration of multidimensional cancer genomics datasets.[18,19]

Mutations and DNA copy number data, mRNA expression data, and deidentified clinical and survival data were extracted from cBioPortal employing the TCGA breast cancer (BRCA) Pan-Cancer Atlas dataset (n=1072, 12 male patients were excluded from the analyses) (http://www.cbioportal.org/; http://www.cancer.gov/tcga).

Correlations between breast cancer patient survival and *H4R* expression (probe set: 221170_at) were further analyzed by KM plotter, mRNA gene chip (http://kmplot.com).[20] "Auto select best cutoff" and all datasets were chosen in the analysis. Patient cohorts with high and low H4R expressing tumors were compared by a Kaplan–Meier survival plot, and the hazard ratio with 95% confidence intervals and log-rank p value were calculated.

Patient Selection

Thirty female patients with TNBC that underwent breast surgery at the British Hospital of Buenos Aires, Argentina, between January 2005 and December 2013 were retrospectively studied using archived paraffin-embedded tumor tissue specimens. The clinical, demographic, and histopathologic data recorded are described in Table 1. Survival data were available for 23 patients in a period of 24 months and during that period, 5 patients died due to breast cancer and 18 of them were alive. The follow-up was not available in seven patients. A great majority of the patients (n=25; 83.3%) underwent additional therapies (adjuvant chemotherapy and/or radiotherapy).

Male breast cancer, benign lesions, and non-epithelial breast tumors were excluded. Poorly preserved samples with extensive necrosis were not used in this study. The study was conducted in accordance with the Declaration of Helsinki and approved by the institutional review board of the British Hospital (CRIHB #925).

Cell Culture and Immunostaining

The human MDA-MB-231 TNBC and MCF-7 luminal breast cancer cells and HEK293 cells (human cell line originally derived from human embryonic kidney cells) (American Type Culture Collection; VA) were cultured in RPMI 1640 supplemented with 10% v/v FBS, 0.3-g L-1 glutamine, and 0.04-g L-1 gentamicin (Gibco BRL;

Table 1. Clinicopathological Characteristics of the TNBC Patients.

Population	Variables	Patient Number, *N*=30	Proportion (%)
Clinical features			
Age (years)	Mean/Range	52.3 (25–69)	
Tumor laterality	Right breast	12	40
	Left breast	18	60
Tumor focality	Unifocal	21	70
	Multifocal	9	30
Type of surgery	Breast conserving surgery	23	76.7
	Mastectomy	7	23.3
Pathological features			
Size (cm)	Mean/Range	2.06 (0.4–4.5)	
Histopathology	Invasive ductal carcinoma	26	86.7
	Other type	4	13.3
Histologic grade	High grade	21	70
	Low grade	9	30
Lymphovascular invasion	No	21	70
	Yes	9	30
Accompanying in situ pattern	No	8	26.7
	Yes	22	73.3
Lymph node metastases	No	18	60
	Yes	12	40
Ki67	≤20%	6	20
	>20%	24	80
Histologic stage	I–II	25	83.3
	III	5	16.7
Recurrence	No	24	80
	Yes	6	20

Abbreviation: TNBC, Triple-negative breast cancer.

NY). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The procedures were previously described.[12,21] Briefly, cells were cultured on glass coverslips into 12-well plates for 24 hr, and they were then fixed with 4% formaldehyde and endogenous peroxidase activity was blocked with 3% hydrogen peroxide (v/v) in distilled water. After blocking, cells were incubated overnight in a humidified chamber at 4°C with primary rabbit anti-H4R (1:100, cat. no PA5-33850; Invitrogen, ThermoFisher Scientific). Immunoreactivity was detected by using the Peroxidase Vectastain Elite ABC-HRP universal kit, according to the manufacturer's instructions. Cells were counterstained with hematoxylin and were visualized using light microscopy (Axiolab Karl Zeiss; Göttingen, Germany). HEK293 cells were used as negative control,[21] while MDA-MB-231 and MCF-7 cells were employed as positive controls of H4R expression.[12,14] The expression of H4R was further assessed in breast cancer cells by flow cytometry as previously described.[12] We used a primary rabbit anti-H4R antibody (1:100, cat. no ab97487; Abcam) followed by a secondary anti-rabbit antibody conjugated with FITC (1:80, cat. no F0382; Sigma Chemical Co., MO). Samples were run on a BD Accuri C6 flow cytometer (BDB) and data were analyzed using BD Accuri C6 software (BDB).

Histopathological and Immunohistochemical Analyses

Histopathological and immunohistochemical assessments were carried out on formalin-fixed paraffin-embedded tissue sections, which included representative samples of carcinomas and adjacent normal breast tissue. The diagnosis was established on hematoxylin and eosin sections by two board-certified pathologists separately. Histological grading and TNM staging (T describes the size of the tumor and any spread of cancer into nearby tissue; N describes spread of cancer to nearby lymph nodes; and M describes metastasis) were determined according to the World Health Organization (WHO) classification.[22,23] Tumors were categorized into low grade (grades 1 or 2) and high grade (grade 3), as previously described.[24]

The blocks were cut in 5-µm sections and were immunolabeled with rabbit monoclonal antibodies directed against ER (clone SP1, 1:100; Cell Marque), PR (clone Y85, 1:30; Cell Marque), HER2 (Her2/Neu,

clone SP3, 1:300; Cell Marque), and Ki67 (clone SP6, 1:200; Cell Marque), using an automated immunohistochemical staining equipment, according to the manufacturer's guidelines (Benchmark XT; Ventana), and the standardized and approved procedure of the British Hospital Institution. Immunoreactivity was assessed blinded to clinicopathological data, using a semiquantitative scoring system. The immunostaining scores for ER, PR, and the algorithm for HER2 scoring were determined according to the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) guidelines. Nuclear and membranous expression was considered positive for ER/PR and HER2, respectively. The threshold for the definition of TNBC was <1% immunopositivity of either ER or PR, and an immunoscore of 0 or 1+ for HER2 expression or 2+ in the absence of amplification by fluorescent in situ hybridization.[4,22–26]

H4R Immunostaining and Scoring

The expression of H4R in tumors and peritumoral tissue was evaluated by immunohistochemical staining as it was previously described.[14] Briefly, after deparaffinization, the specimens were heated in a microwave in sodium citrate buffer (10 mM, pH 6.0) for antigen retrieval. After blocking, specimens were incubated with primary rabbit anti-H4R polyclonal antibody directed against the first cytoplasmic domain of human H4R (1:100, cat. no PA5-33850; Invitrogen) antibodies overnight in a humidified chamber at 4°C. Immunoreactivity was detected by using the Peroxidase Vectastain Elite ABC-HRP universal kit, according to the manufacturer's instructions. Preimmune serum of the same animal species in which the secondary antibody was developed was used for blocking, and to replace the primary antibody to detect nonspecific binding of the secondary antibodies (PK-6200; Vector Laboratories, CA). All specimens were processed following identical and standardized staining procedures.

The H4R immunoreactivity score was obtained by multiplying the intensity (negative, 0; weak, 1; moderate, 2; and strong, 3) by the percentage of stained cells. H4R expression was considered to be "positive" if at least 5% of cell specimens showed membranous and/or granular cytoplasmic staining. All the evaluations were performed by consensus agreement of at least two specialized pathologists. Immunocompetent cells were considered internal positive controls in the specimens.[11] Visualization was performed with an optical microscope Leica ICC50 HD (Wetzlar, Germany). Photographs were taken at 100× and 400× magnification with Leica camera (Germany) and visualized with Leica LAS EZ software (v3.1.0; Leica Microsystem, Switzerland).

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism v7.00 (San Diego, CA). Mann–Whitney nonparametric test was used to compare average scores. Wilcoxon matched-pair signed-rank test was used for the statistical analysis of differences in protein expression between tumor-adjacent peritumoral normal tissue pairs. For determination of the association among different variables, Spearman's rho correlation coefficients and two-tailed significance were determined. Log-rank test and Gehan–Breslow–Wilcoxon test were performed for Kaplan–Meier survival. All statistical tests were two-sided, and a p value <0.05 was significant.

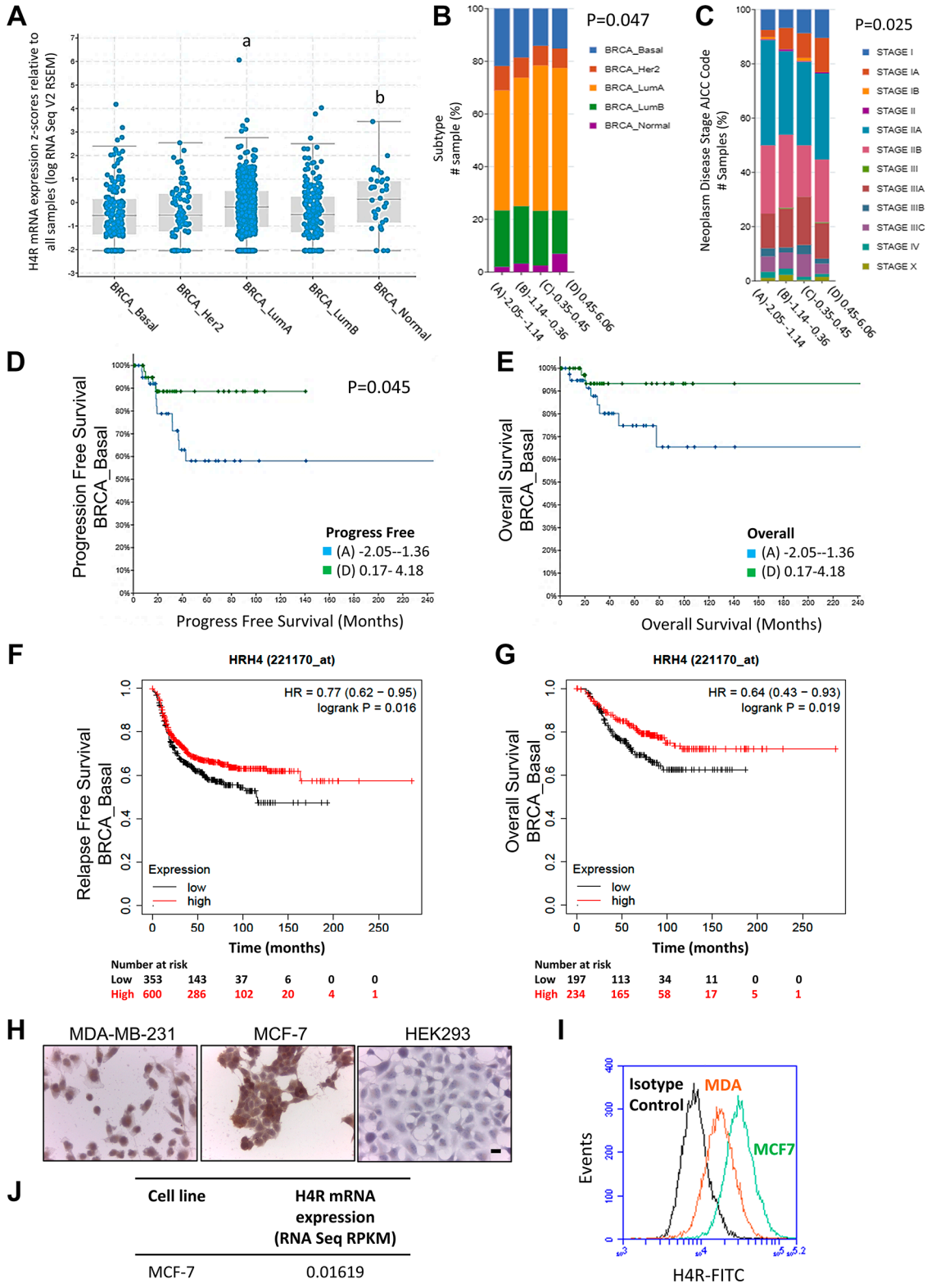
Results

H4R Expression in Human Breast Cancer Samples

We have previously demonstrated the functional expression of H4R in TNBC experimental models in which H4R ligands showed antitumoral potential.[12–17] However, the evidence of H4R expression and its role in human TNBC cancer progression has remained insufficient.

The potential clinical relevance of H4R in TNBC/basal-like tumors was assessed at a large scale by means of the genomic expression and clinical data obtained from publicly available datasets. Analyses of TCGA Pan-Cancer Atlas dataset[27,28] show that H4R mRNA expression was lower in the aggressive basal-like tumors compared with the more favorable clinical outcome luminal A (p=0.028) and normal breast-like tumors (p=0.018) (Fig. 1A). Tumors were split into quartiles based on *H4R* expression, and cancer subtypes, staging, and survival were investigated.

The highest quartile of *H4R* expression had a greater proportion of luminal A and normal breast-like tumors with lower proportion of basal-like compared with the lowest quartile (Fig. 1B). Likewise, the evaluation of *H4R* gene alterations (including deletions, amplifications, and mutations) frequency in the different breast cancer subtypes obtained from cBioPortal web resource revealed that the vast proportion of gene alterations were observed in basal-like tumors (Supplemental Fig. 1A). Interestingly, a significant reduced survival was observed in the group of breast cancer patients with H4R with at least one type of gene alteration (Supplemental Fig. 1B). Furthermore, higher levels of H4R mRNA expression were observed in early-stage breast cancer (Fig. 1C).



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Cell line	H4R mRNA expression (RNA Seq RPKM)
MCF-7	0.01619
MDA-MB-231	0.00562

Figure 1. *(continued)*

Figure 1. Bioinformatic analyses of the expression of *H4R* in breast cancer. mRNA expression levels of *H4R* were obtained from breast cancer datasets at the cBioPortal for Cancer Genomics (TCGA Pan-Cancer Atlas). (A) H4R mRNA expression in different breast cancer (BRCA) subtypes. Box plots show the expression levels as log-transformed mRNA expression z scores compared with the expression distribution of all samples (RNA Seq V2 RSEM). BRCA_Basal (n=171), BRCA_Her2 (n=78), BRCA_LumA (Luminal A, n=499), BRCA_LumB (Luminal B, n=197), BRCA_Normal (normal breast-like, n=36). a: p=0.028 vs. BRCA_Basal; b: p=0.018 vs. BRCA_Basal. Kruskal–Wallis test and Dunn's multiple comparisons test. (B) Percentage of samples with different breast cancer subtypes based on *H4R* expression quartiles. Chi-squared test, p=0.047. (C) Percentage of samples with different neoplasm disease stages American Joint Committee on Cancer (AJCC) code based on *H4R* expression quartiles. Chi-squared test, p=0.025. A (n=267): the lowest quartile, −2.02 to −1.14; B (n=268): −1.14 to −0.36; C (n=267): −0.35 to 0.45; D (n=268): the highest quartile, 0.45 to 6.06 (log RNA Seq V2 RSEM). (D, E) Kaplan–Meier plots comparing the clinical outcomes of patients with high vs. low *H4R* expressing basal-like tumors (TCGA Pan-Cancer Atlas). (D) Progression-free survival and (E) OS were evaluated for the lowest (A, n=42: −2.05 to −1.36 log RNA Seq V2 RSEM) and the highest (D, n=43: 0.17 to 4.18 log RNA Seq V2 RSEM) *H4R* expression quartiles. Mantel–Cox (log-rank test). Progression-free survival: p=0.045. OS: p=NS. (F, G) Kaplan–Meier plots comparing the clinical outcomes of patients with high vs. low *H4R* expressing basal-like tumors (Kaplan–Meier Plotter). (F) Relapse-free survival and (G) OS. Red line: patients with expression levels above the median; black line: patients with expression levels below the median. Mantel–Cox (log-rank test). Relapse-free survival: p=0.016. OS: p=0.019. (H–J) H4R expression in human cancer cell lines. (H) Immunocytochemical detection of H4R in MDA-MB-231 and MCF-7 breast cancer cells. HEK293 cells were used as a negative control. 400× original magnification. Scale bar = 20 µm. (I) Immunofluorescence of H4R was evaluated by flow cytometry. Representative histograms are shown. (J) H4R mRNA expression (RNA Seq RPKM) obtained at cBioPortal (Cancer Cell Line Encyclopedia, Broad 2019).[29] Abbreviations: H4R, histamine H4 receptor; TCGA, The Cancer Genome Atlas; OS, overall survival; NS, not significant.

These results suggest that the H4R seems to be particularly impaired in basal-like breast cancer. To deepen its role in tumor biology in this subtype and illustrate the potential prognostic value of H4R, survival rates based on progression or mortality were evaluated in basal-like breast cancer stratified by H4R low and high expression. Higher levels of H4R mRNA expression were significantly associated with improved progression-free survival, and a non-significant increase in the overall survival (OS) in basal-like breast cancer (Fig. 1D and E). Findings were confirmed using the Kaplan–Meier plotter database to evaluate the survival of basal-like cancer patients (probe 221170_x_at for H4R). The result indicated that a high level of H4R was significantly associated with improved relapse-free survival [hazard ratio (HR) 0.77, p=0.016, Fig. 1F] and OS (HR 0.64, p=0.019, Fig. 1G) in basal-like cancer patients.

Although there is around an 80% overlap between triple-negative and intrinsic basal-like breast cancer subtype, the basal-like classification is defined via gene expression analysis and to date is limited to the research setting. TNBC phenotype refers to the immunohistochemical classification of breast tumors lacking ER, PR, and HER2 protein expression and it is currently a reliable surrogate in the clinical setting.[3,4,30]

To validate the transcriptomic data, we next evaluated the immunohistochemical protein expression of H4R in a small cohort of patients with TNBC. The specificity of the antibody was checked using HEK293 cells, which do not endogenously express H4R.[21] As we have previously reported,[12,14] we detected H4R in human MCF-7 and MDA-MB-231 cells by immunocytochemistry, which served as positive controls (Fig. 1H). Interestingly and in line with the results obtained of the expression of H4R in patient datasets, MCF-7 luminal-like breast cancer cells seemed to express higher levels of H4R compared with MDA-MB-231 TNBC cells. This upregulation of H4R in MCF-7 cells was confirmed by a semiquantitative flow cytometric analysis (Fig. 1I), and additionally investigating H4R mRNA expression (Cancer Cell Line Encyclopedia at cBioPortal) (Fig. 1J).

Expression of H4R in TNBC and Matched Histologically Normal Breast Tissue and Its Association With Clinicopathological Features

Next, we examined the relationship between H4R protein expression and its association with disease characteristics. Thirty TNBC specimens were analyzed in this study. Patient characteristics are summarized in Table 1. The clinicopathological features in the tumor samples were compared in the patient cohort. Negative nodal disease and unifocal TNBC were associated with a favorable prognosis (Supplemental Table 1).

H4R immunostaining shows a membranous and granular cytoplasmic pattern in the TNBC samples, which exhibited different levels of expression (Fig. 2A, Supplemental Fig. 2).

Twenty-one of the 30 tumors (70%) exhibited positive immunostaining for H4R with a score ranging between 5 and 180, while 9 tumors showed negative expression (Fig. 2A to C). In addition, the expression of H4R was analyzed in the peritumoral normal tissue defined as the histologically normal tissue adjacent to the tumor. A positive H4R immunostaining was observed in 22 of the 26 specimens (85%), exhibiting

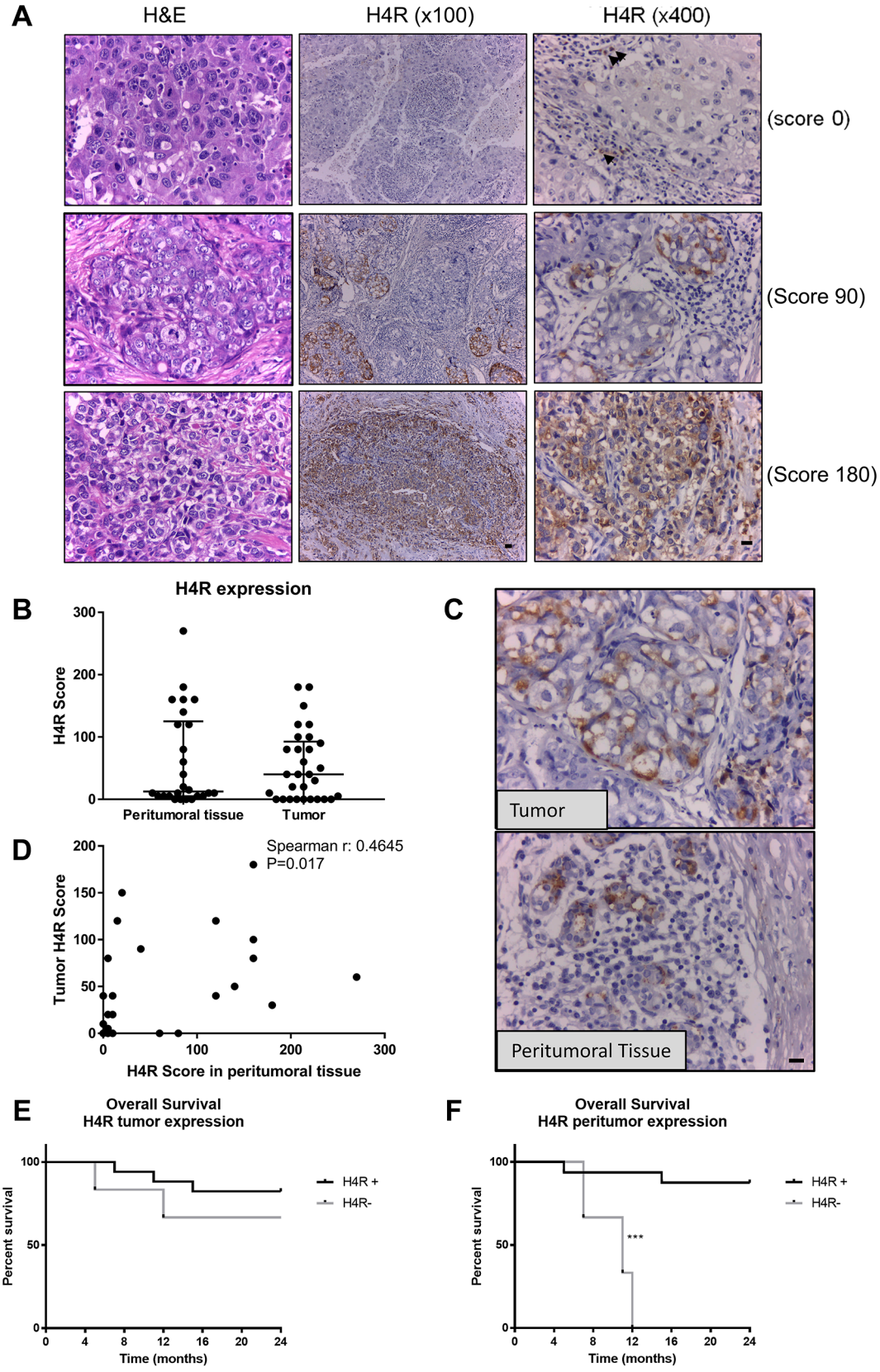

Figure 2. *(continued)*

Figure 2. H4R expression in tumoral and peritumoral tissue of TNBC patients. (A) Representative TNBC samples stained with hematoxylin and eosin (H&E) and H4R immunostaining. All corresponded to high-grade invasive ductal carcinomas with variable amounts of ductal differentiation and numerous atypical mitoses. The score of H4R immunostaining was obtained by multiplying the intensity (negative, 0; weak, 1; moderate, 2; and strong, 3) by the percentage of stained cells. Negative expression of H4R (score 0), arrows indicate positive immunocompetent cells. Positive membranous and granular cytoplasmic staining of H4R with scores of 90 (3X30) and 180 (3X60) are shown. 100× and 400× original magnification. Scale bar = 20 μm. Representative pictures of the scale of H4R intensities are shown in Supplemental Fig. 2. (B) H4R immunostaining score in tumor (score range: 5–180) and peritumoral breast tissue (score range: 5–270). (C) Similar H4R expression was seen in neoplastic cells (above) in comparison to normal ducts of the peritumoral breast lobules (below). H4R expression was always membranous and cytoplasmic. 400× original magnification. (D) Spearman's positive correlation between H4R expression in tumoral and peritumoral tissue. (E, F) Kaplan–Meier survival curves according to the expression of H4R (follow-up: 24 months). (E) Log-rank and Mantel–Cox test: χ^2 (chi-square) = 0.001, p=NS, and the Gehan–Breslow–Wilcoxon test: $\chi^2 = 0.1656$, p=NS. (F) Log-rank and Mantel–Cox test: $\chi^2 = 14.34$, $p<0.001$, and the Gehan–Breslow–Wilcoxon test: $\chi^2 = 12.78$, $p<0.001$. Abbreviations: H4R, histamine H4 receptor; TNBC, Triple-negative breast cancer; NS, not significant.

a score ranging from 5 to 270 (Fig. 2B and C). Interestingly, there was a moderate positive correlation between the expression of H4R in the tumoral and peritumoral tissue (Fig. 2C and D).

Considering that normal peritumoral tissue may exhibit alterations at the molecular level that could be associated with cancer risk,[31–33] both types of samples were investigated. Elevated expression of H4R was demonstrated in relation to unifocal TNBC, which was significant in peritumoral histopathologically normal tissue (Table 2). No significant differences were detected between the H4R expression in tumor and peritumoral tissue and the histopathological grade, size, nodal status, or the high proliferation index measured by Ki67 (Table 2). However, a negative correlation between H4R expression in peritumoral tissue and the number of lymph node involvement was found (Spearman r: –0.4793, p=0.015).

Survival studies showed that patients with H4R positivity have increased OS compared with H4R-negative specimens, which was significant considering H4R staining in peritumoral tissue (Fig. 2E and F).

Discussion

TNBC represents a major clinical therapeutic challenge. Recent data demonstrate the expression of H4R and its pathophysiological role in cancer, representing a potential molecular target for cancer therapeutics.[11,13,14,17,34] This study provides evidence of the expression of H4R in TNBC and its potential association with prognosis.

TCGA is a publicly available database that shows the most important genomic changes in tumors of 33 types of cancers from thousands of patients, which notably contributes to accelerating our knowledge of the molecular basis of cancer with impacts in both cancer prevention and treatment. Using TCGA data, we have recently described the *H4R* gene expression in different types of tumors compared with matched-normal tissues. Depending on the cancer type, *H4R* seemed to be downregulated (e.g. colon adenocarcinoma, breast-invasive carcinoma, bladder urothelial carcinoma), upregulated (e.g. hepatocellular, esophageal, and kidney cancers), or unchanged (e.g. lung adenocarcinoma) compared with normal tissue.[11,17,21,35–39]

In this study, we analyzed a large transcriptomic dataset associated with clinical features (TCGA Pan-Cancer Atlas) by means of cBioPortal for Cancer Genomics. The analysis of the H4R mRNA expression in different molecular subtypes of breast cancer demonstrated that H4R is downregulated in basal-like breast cancer compared with luminal A breast cancer and normal breast-like tumors, both favorable subtypes in terms of prognosis.[3,30] In agreement with these results, luminal MCF-7 cells showed higher H4R expression compared with basal-like MDA-MB-231 breast cancer cells.

An inverse relationship was evidenced when comparing the expression of *H4R* according to the neoplasm disease stage. A higher proportion of stage I non-spread breast cancer showed higher levels of *H4R* expression. In addition, the study of the alteration frequency of *H4R* gene in the breast cancer subtypes showed different percentages of alterations depending on the cancer subtype. The higher frequency of alterations, that include deletions and amplifications of the *H4R* gene, was observed in basal-like breast cancer compared with the other subtypes. Interestingly, survival analysis showed improved disease-free survival in breast cancer patients without H4R gene alterations. Genomic alterations of this receptor in different cancer types have been described[17]; however, their role in carcinogenesis and in the response to therapeutics is completely unknown and deserves to be studied. These findings suggest that H4R may play a crucial role in breast cancer biology and progression, especially in the aggressive basal-like breast cancer. Therefore, Kaplan–Meier curves for basal-like breast cancer patients were stratified by H4R expression. The higher expression of H4R was associated with better

Table 2. H4R Expression According to Different Clinicopathological Parameters in TNBC Patients.

		H4R Expression			
		Tumor Score		Peritumoral Score	
Clinicopathological Parameter		Median (IQR)	*p* Value	Median (IQR)	*p* Value
Tumor focality	Unifocal	50 (12.5–95)	NS	60 (7.5–160)	0.011
	Multifocal	0 (0–80)		5 (0–17.5)	
Histologic grade	High	40 (0–82.5)	NS	10 (5–125)	NS
	Low	35 (0–142.5)		17.5 (1–140)	
LN metastases	No	50 (3.7–105)	NS	80 (5–160)	NS
	Yes	25 (0–72.5)		10 (1–48.7)	
Ki67	≤20%	50 (15–110)	NS	140 (62.5–170)	NS
	>20%	40 (0–87.5)		10 (5–55)	
Tumor size	>2 cm	35 (0–115)	NS	10 (5–35)	NS
	≤2 cm	45 (0–80)		100 (5–160)	

Abbreviations: H4R, histamine H4 receptor; LN, lymph node; NS, not significant; IQR, interquartile range; TNBC, Triple-negative breast cancer. Mann–Whitney's test.

survival clinical outcomes based on both progression and mortality events.

In line with these results, evidence from independent research groups demonstrated that potent H4R agonists reduced cell proliferation and events involved in the metastatic cascade in different cancer types.[11,14,17,21,34,36,37,39] Therefore, H4R might contribute to improvements in cancer treatment in terms of a targeted therapy.

To corroborate the bioinformatic analyses, we investigated the protein expression of H4R in TNBC samples, according to pathology-based classification. To our knowledge, this study is the first to assess the immunohistochemical H4R expression specifically in human TNBC samples. Membranous and cytoplasmic H4R immunostaining was detected in 70% of TNBC samples. The expression of H4R was further demonstrated in the histologically normal breast tissue located adjacent to the carcinoma. The analysis of the expression of H4R in the peritumoral breast tissue revealed no significant differences with its expression in tumor epithelial cells, and a moderate positive correlation between the H4R score in the tumoral and peritumoral tissues.

Numerous reports suggest that histologically normal tissue adjacent to breast cancer may harbor molecular alterations, which could support tumorigenesis.[31–33,40–46] In this connection, the identification in routine breast biopsies of a molecular marker in appearing normal tissue at risk for malignant transformation may have useful potential clinical application.[33,45,46] H4R expression is inversely correlated with the number of regional lymph node metastases in peritumoral tissue. The number of involved axillary lymph nodes remains the dominant predictor of prognosis in breast cancer, overwhelming other factors and conditioning the decision of the adjuvant systemic treatment.[47–50] Furthermore, multifocal TNBC was associated with reduced H4R expression in peritumoral tissue. Although a link between tumor focality and prognosis is not well understood, some studies show that multifocal lesions, when more than one tumor of the same origin arises in the same area of the breast, could be associated with a higher risk of recurrence.[47] In the absence of lymph node metastases, tumor size and its histological grade, or proliferative index, contribute to sorting patients into groups according to cancer risk.[47] However, these parameters were neither prognostically important in terms of survival nor differentially modulated by H4R in our small patients' cohort.

Kaplan–Meier curves for OS of patients with TNBC were obtained according to the presence or absence of H4R in both tumor and peritumoral tissue. Patients with H4R expression had significantly better OS than those with undetectable levels of H4R just in peritumoral tissue. Presented data indicate that H4R expression in TNBC seems to be reduced or absent in more aggressive or disseminated tumors. We hypothesize that impairment of H4R expression in tumor-adjacent, histologically normal breast tissue could be present in breast epithelium as an early molecular change before clinical or pathological evidence of the neoplasm. Ongoing experimental studies are aimed at investigating the H4R expression in histologically normal tissue of breast cancer patients compared with normal epithelium of women without breast cancer as an approach to better understand the significance of H4R in carcinogenesis.

Our research has numerous limitations that should be described. First, the study was limited by a small sample size of a single institution. Some patients performed their treatments outside the institution or discontinued it, preventing follow-up data during a long period. Due to the small sample size, a meaningful statistical analysis of the correlation between H4R score and some clinicopathological parameters could not be possible. In addition, multivariate analysis is necessary to identify H4R as a potential independent predictor of clinical outcomes.

In conclusion, H4R transcriptomic data together with the immunohistochemical studies suggest that the H4R might represent a novel prognostic factor associated with aggressiveness and patient survival in TNBC, which could complement routine histopathological analysis. Furthermore, the detection of H4R in TNBC samples is clinically relevant considering that it could represent a promising therapeutic target for this aggressive and difficult-to-treat type of breast cancer. In this sense, this study serves as important data for the initiation of further studies to understand the significance of H4R in breast cancer biology and prognosis in large patient cohorts.

Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

Conceived and designed the experiments: DS, JLU, and VAM. Performed the experiments: DS, MATD, MBN, IAO, FV, and PD. Analyzed the data: DS and VAM. Contributed reagents/materials/analysis tools: JLU, AI, GE, and VAM. Wrote the article: DS and VAM. All authors approved the article.

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