Kiss-1/GPR54 Protein Expression in Breast Cancer

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Abstract. *Background: Numerous studies have shown that the Kiss-1 gene countervails the metastatic aptitude of several cancer cell lines and solid-tumor neoplasias. However, there still remains ambiguity regarding its role in breast cancer and literature has arisen asserting that Kiss-1 expression may be linked to an aggressive phenotype and malignant progression. Herein, we investigated the protein expression of Kiss-1 and its receptor GPR54 in breast cancer tissues compared to non-cancerous mammary tissues. Materials and Methods: Paraffin-fixed cancer tissues from 43 women with resected breast adenocarcinomas and 11 specimens derived from women suffering from fibrocystic disease, serving as controls, were immunostained with Kiss-1 and GPR54 antibodies. Results: Kiss-1 and GPR54 protein expression levels were significantly higher in breast cancer compared to fibrocystic tissues ($p<0.05$). No significant correlation was established between Kiss-1 or GRP54 expression and tumor grade, tumor size, lymph node positivity, histological type or ER status. Kiss-1 expression significantly and positively correlated with GPR54 expression in both breast cancer and fibrocystic disease specimens. Conclusion: Kiss-1/GPR54 expression was found to be significantly higher in breast cancer compared to non-malignant mammary tissues.*

The development of distant metastasis requires for complex sequential steps that include breakage of normal architectural boundaries, invasion of cancer cells into the vascular and lymphatic system, directed migration, re-invasion, attachment and proliferation at the peripheral sites, and, finally, the ability to induce angiogenesis in order to provide the growing tumor with essential nutrients (1, 2). Inhibition of any one of these steps can disarrange the metastatic cascade and thus reduce the potential of secondary tumor development. The metastatic process is tightly-orchestrated by an interplay between metastasis-suppressor and metastasis-promoter genes. Metastasis suppressor genes, which produce protein factors that hinder the metastatic capacity without affecting tumorigenicity (3), have therefore emerged as key research targets for metastasis-directed therapy.

Kiss-1 has been isolated as a novel metastasis suppressor gene when subtractive hybridization and differential display techniques were employed to identify differences in gene expression patterns between metastatic melanoma cancer cells and hybrid clones that were rendered non-metastatic by microcell-mediated transfer of an intact copy of chromosome 6 (4, 5). The *Kiss-1* gene maps to the long arm of chromosome 1 (1q32-q41) and incorporates 4 exons, with only the last two being partially translated (6). *Kiss-1* gene expression seems to be regulated by genes located on chromosome 6, since loss of heterozygosity of 6q16.3-q23 has been associated with loss of Kiss-1 expression in melanoma cells (7).

The *Kiss-1* gene sequence predicts a largely hydrophobic 145-amino acid protein which harbors a protein kinase C phosphorylation domain, a putative secretory signal, a polyproline-rich region (SH3 ligande) and a series of motifs for post-translation modifications (4, 8, 9). The 145-amino acid precursor protein is proteolytically-processed to generate a series of overlapping N-terminally-truncated peptides which are collectively designated as "kisspeptins". Four biologically-active cleavage derivatives have been characterized, kisspeptin-54 (KP-54 or metastin, comprising residues 68-121), kisspeptin-14 (KP-14), kisspeptin-13 (KP-13) and kisspeptin-10 (KP-10) (9, 10,

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11). All kisspeptin derivatives share a common 10-amino-acid sequence at the C-terminal tail (H-Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2), which comprises of the canonical Arg-Phe-NH2 suffix of the RF-amide peptide family (9, 11). The C-terminal decapeptide is the essential pharmacophore required for functional receptor interaction (8, 9, 11), with KP-10 reported to exhibit even higher receptor affinity and biopotency compared to the extended forms of the peptide (8). Kisspeptins mediate their effect by binding to at least one known endogenous receptor, GPR54, which belongs to the $G_{q/11}$ subfamily of G-protein coupled receptors (GPCRs) (8).

Several bioassays have shown that *Kiss-1* gene products impede invasion, motility and chemotaxis of several cancer cell lines *in vitro* (5, 9, 11, 12, 13). The coupling of kisspeptin to GPR54 triggers PIP(2) hydrolysis, Ca(2+) mobilization and arachidonic acid release (9), stimulates ERK1/2 and p38 MAP kinase phosphorylation (9), and suppresses matrix metalloproteinase-9 (MMP-9) expression by down-regulating NF-kappa B binding to the promoter (14). Kisspeptin has also been reported to induce excessive stress fiber and focal adhesion formation through activation of the Rho sub-family of G proteins (9). Furthermore, there is an association between loss of Kiss-1 expression and loss of E-cadherin expression (15), a gene known to play an important anti-metastatic role in several cancers including breast (16) and bladder (17). Finally, DNA microarray technology revealed that GPR54 up-regulates a number of pro-apoptotic genes and promotes cell-cycle arrest in human breast cancer cells MDA-MB-435S (18).

In non-malignant tissues, Kiss-1 expression has been shown to be particularly abundant in the placenta (8), contributing to trophoblast invasion during pregnancy (10, 19, 20, 21), followed by a widespread expression in several central nervous system regions and moderate-to-weak expression in the testis, pancreas, liver, intestine, kidney, lungs and prostate (4, 8, 11). This tissue distribution pattern is compatible with mRNA localization of GPR54, which is highly expressed in the placenta and central nervous system, and less prominent in intestine, kidney, lungs, and prostate (4, 8, 11). The striking similarities between metastatic cancer cells and invading cytotrophoblast cells (22, 23, 24, 25, 26), along with the fact that Kiss-1 and GPR54 are predominantly expressed in the placenta, suggest that Kiss-1 and GPR54 may be components of a biological mechanism that fine-tunes both processes.

Kiss-1 and GPR54 expression also has been investigated in a variety of cancers including gestational trophoblastic disease (27), melanoma (4, 7), breast cancer (28, 29), hepatocellular carcinoma (30), pancreatic cancer (31), gastric carcinoma (32), esophageal carcinoma (33), papillary thyroid cancer (34), bladder cancer (15), ovarian cancer (35, 36), prostate cancer (37) and pheochromocytoma (38). The Kiss-1/GPR54 system has also been implicated in the pathophysiology of endometriosis (39). In most malignancies, the *Kiss-1* gene seems to act as an anti-metastatic agent and loss of Kiss-1 expression is associated with tumor progression and advanced disease. In breast cancer, however, the role of kisspeptin remains elusive due to limited and conflicting data, and it is possible that increased Kiss-1 expression correlates with disease progression and poor patient prognosis in this particular type of cancer. The present study aimed at determining Kiss-1 and GPR54 protein expression in breast cancer tissues compared to non-malignant mammary tissues.

Materials and Methods

Tissues and immunohistochemical methods. Forty-three formalin-fixed and paraffin-embedded tissue specimens derived from patients with resected breast adenocarcinomas were recruited from our tissue bank. The breast cancer specimens consisted of 32 ductal carcinomas, and 11 lobar carcinomas. Eleven non-malignant mammary gland tissues served as controls and were obtained from 10 women suffering from fibrocystic disease and one woman diagnosed with benign phyllodes tumor. The cancer group comprised of women ranging in age from 30 to 99 years (average age 62 years). Histological classification of samples utilized for immunohistochemistry is described in Table I.

The Bondmax automated system (Leica Microsystems, New Castle, UK) was used for the immunohistochemical staining of paraffin sections, using the Kiss-1 (FL-145) monoclonal antibody and the anti-GPR54 polyclonal antibody at 1:150 and 1:100 dilution respectively. Villous and extravillous trophoblast of the placenta were used as positive controls for both antibodies. Furthermore, negative controls were performed by omitting the primary antibody. Cytoplasmic expression was evaluated for Kiss-1 and cytoplasmic/membranous expression for GPR54 respectively. Immunohistochemical expression of stained proteins was classified as either grade 1 (weak intensity), grade 2 (moderate intensity) or grade 3 (strong intensity). The H scoring system, incorporating both intensity and distribution of staining (40, 41), was used for semi-quantitative analysis of protein immunoreactivity. More specifically, the percentage of positive cells was measured in every section and multiplied by 1, 2 and 3, respectively (grade 1 score=percentage with grade 1 expression ×1; grade 2 score=percentage with grade 2 expression ×2; grade 3 score=percentage grade 3 expression ×3). A total score between 0 and 300 was obtained for each case (total score=grade 1 score + grade 2 score + grade 3 score).

Relevant clinicopathological data were retrieved from archived material. Tumors were staged according to the tumor-node-metastasis (TNM) system recommended by the International Union Against Cancer (1997). Grade was assigned by the Bloom–Richardson method, Nottingham modification. Approval of the study was acquired from the local institutional ethics committee.

Statistical analysis. All statistical analyses were performed using R (Foundation for Statistical Computing, Vienna, Austria) (42). The Kolmogorov-Smirnov test was applied for analysis of variance in all continuous variables and the choice of methods for statistical testing of continuous variables was based on whether the data permitted parametric or non-parametric analysis. Differences

Table I. *Relationships between Kiss-1/GPR54 status and other clinicopathological variables of the study.*

Variable		Association with Kiss-1 expression	Association with GPR54 expression
Age[a]	62 years (30-99)	r=0.226; p=0.1[c]	r=0.281; p=0.039[c]
Histological type[b]			
Ductal	32 (74.4%)	p=0.180[d]	p=0.902[d]
Lobar	11 (25.6%)		
Tumor grade[b]			
1	2 (5.55%)		
2	9 (25.00%)	p=0.442[e]	p=0.417[e]
2-3	4 (11.11%)		
3	21 (58.33%)		
Lymph node metastasis[b]			
Negative	18 (48.64%)	p=0.111[d]	p=0.750[d]
Positive	19 (51.36%)		
ER[b]			
Negative	14 (34.14%)		
1	9 (21.95%)	r=0.209;	r=0.084;
2	7 (17.07%)	p=0.178[c]	p=0.594[c]
3	11 (26.82%)		
PR[b]			
Negative	17 (41.47%)		
1	7 (17.07%)	r=–0.002;	r=–0.050;
2	4 (9.75%)	p=0.988[c]	p=0.749[c]
3	13 (31.71%)		
cerb2[b]			
Negative	16 (39.02%)		
1	9 (21.96%)	r=0.068;	r=0.304;
2	5 (12.20%)	p=0.665[c]	p=0.048[c]
3	11 (26.82%)		
p53[b]			
Negative	26 (68.43%)		
1	7 (18.42%)	r=–0.079;	r=0.043;
2	2 (5.26%)	p=0.616[c]	p=0.786[c]
3	3 (7.89%)		
Ki67[b]			
Negative	10 (28.57%)		
1	8 (22.87%)	r=0.046;	r=0.043;
2	4 (11.42%)	p=0.616[c]	p=0.786[c]
3	13 (37.14%)		
Tumor size			
Mean[a]	3.89 cm (0.42-13)		
<2 cm[b]	8 (18.61%)	p=0.729[e]	p=0.429[e]
2-5 cm[b]	20 (46.51%)		
>5 cm[b]	15 (34.88%)		

[a]Continuous variable; Data are given as Mean (range); [b]Categorical variable; Data in parentheses represent the percentage of each group; [c]Calculated by Spearman correlation coefficient; [d]Calculated by the Mann-Whitney *U*-test; [e]Calculated by the Kruskal-Wallis test; ER: Estrogen receptor; PR: progesterone receptor.

between two groups were evaluated using the Mann-Whitney *U*-test. Relationships between different continuous variables were assessed by the Spearman correlation coefficient. Statistical significance was set at $p<0.05$.

Results

Distribution and expression of Kiss-1 and GPR54 in cancer and normal mammary tissues. Kiss-1 and GPR54 expression levels were significantly higher in breast cancer tissues compared to fibrocystic disease ($p<0.05$). Among the 54 breast cancer and fibrocystic disease patients, Kiss-1 expression significantly and weakly-positively correlated with GPR54 expression (r=0.337, p=0.013). Representative samples stained with Kiss-1 and GPR54 antibodies are shown in Figures 1 and 2 respectively.

Correlation of Kiss-1 and GPR54 with tumor grade, tumor size and lymph node metastasis. Kiss-1 and GPR54 protein expression were not significantly associated with tumor grade, tumor size or presence of lymph node metastasis ($p>0.05$).

Kiss-1 and GPR54 expression in different histological types. Immunohistochemical data showed no significant differences in Kiss-1 and GPR54 expression between lobar (n=11) and ductal (n=32) breast cancers ($p>0.05$).

Correlation of Kiss-1 and GPR54 with patient characteristics. GPR54 protein expression was significantly and weakly-positively associated with age (r=0.281, p=0.039).

Kiss-1 and GPR54 correlation with hormone receptor status and cell markers. No significant associations between Kiss-1 protein expression and ER, PR, cerb2, p53 or Ki67 were found ($p>0.05$). GPR54 protein expression was only significantly and weakly-positively correlated with cerb2 expression (r=0.304, p=0.048).

Discussion

The class of proteins known as metastasis suppressors can prevent metastases without affecting the growth of the primary tumor and has recently drawn much attention as it may provide useful mechanistic insight for the development of targeted-therapeutic strategies including drug-induced restoration of metastasis suppressor genes and emerged pathways. The *Kiss-1* gene was initially identified as a candidate metastasis suppressor in 1996, when it was found that its expression was differentially up-regulated in C8161 melanoma cells that were rendered non-metastatic by microcell-mediated transfer of an intact copy of human chromosome 6. However, even though the *Kiss-1* gene has been identified as a strong suppressor of metastasis in a variety of cancers, limited and conflicting data have been subjected concerning the intertwined relationships between Kiss-1 system and breast cancer, and its biological role in that particular cancer remains to be elucidated (30, 43, 44). *Kiss-1*

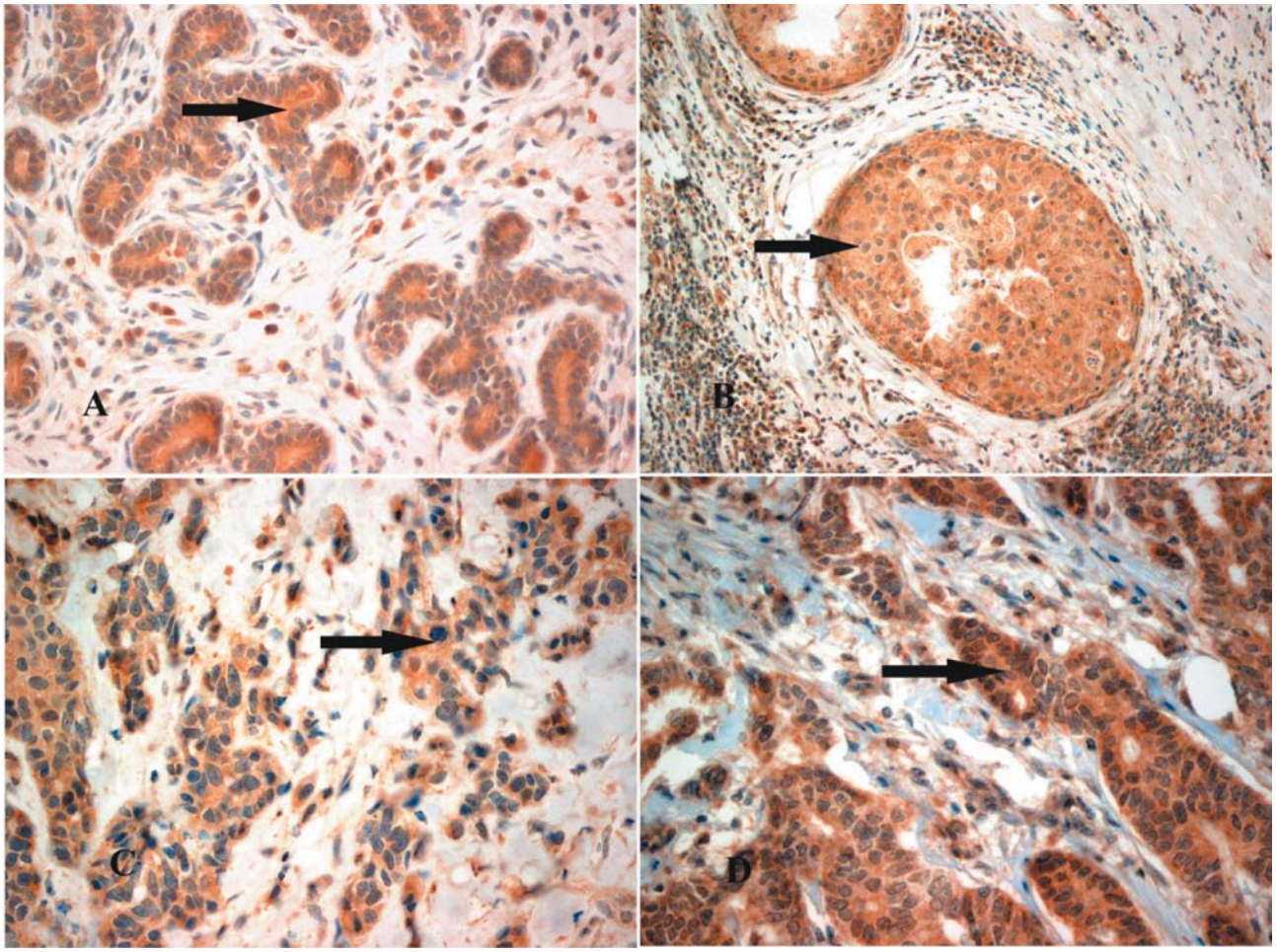

Figure 1. *A. Kiss-1 Immunohistochemical expression (IE) in normal breast epithelium (black arrow). B. Kiss-1 IE in ductal in situ breast carcinoma (black arrow). C. Kiss-1 IE in invasive ductal breast carcinoma (black arrow). D. Kiss-1 IE in invasive ductal breast carcinoma (black arrow). All magnifications ×400, except B ×200.*

was initially postulated as a metastasis suppressor gene in breast cancer when it was reported that inoculation of MDA-MB-435 by *Kiss-1*-transfectant cells into the subaxillary mammary fad pads of female athymic nude mice, suppressed regional lymph node and lung metastasis by at least 95%, without affecting tumorgenicity (29). However another study conducted by Martin *et al*. demonstrated that insertion of *Kiss-1* gene into the MDA-MB-231 breast cancer cell line increased motility and invasiveness and reduced adhesion to matrix, thus conferring a more metastatic phenotype (43). Martin *et al*. also showed that Kiss-1 levels were higher in breast cancer tissues compared to background tissues and increased significantly in node positive tumors compared to node negative tumors (43). Furthermore Kiss-1 expression increased with increasing grade and TNM status and was higher in patients who died from breast cancer than those who remained healthy (43). These results clearly are in conflict with the originally proposed role of Kiss-1 as an inhibitor of metastasis in breast cancer. Marot *et al*. have also reported that ERa-positive tumors resected from post-menopausal women treated with TAM correlated with shorter relapse-free survival (RFS) when they combined high Kiss-1 and GPR54 mRNA tumoral levels, compared to tumors which expressed low levels of both genes (44). The researchers also illustrated that *Kiss-1* and *GPR54* expression in breast cancer cells was negatively regulated by estrogen milieu, with ERa-positive tumors expressing lower Kiss-1 levels compared to ERa-negative tumors (44). In contrast to the above results demonstrating *Kiss-1* as a facilitator of metastasis, Kostadima *et al*. found that Kiss-1 gene was silenced in the vast majority (97%) of 272 resected stage II or III node-positive breast adenocarcinomas (45). Another study also reported that KP-10 can inhibit bone-directed migration of GPR54-positive MCF-7 breast cancer cells (46). It should also be noted that a

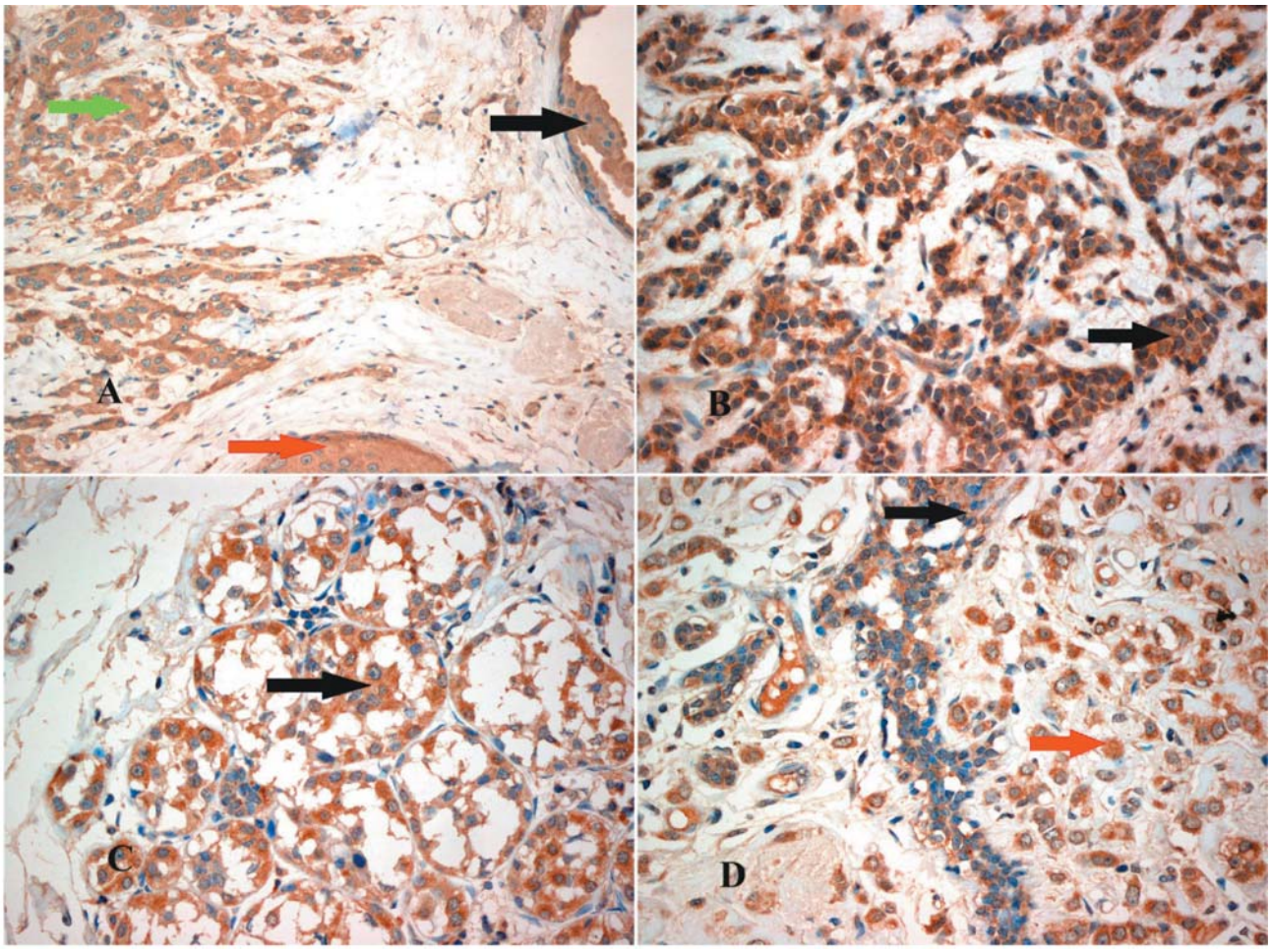

Figure 2. *A. GPR54 Immunohistochemical expression (IE) in apocrine metaplasia (black arrow), ductal in situ breast carcinoma (red arrow) and invasive ductal breast carcinoma (green arrow). B. GPR54 IE in invasive ductal breast carcinoma (black arrow).C. GPR54 IE in lobular in situ breast carcinoma (black arrow). D. GPR54 in normal breast epithelium (black arrow) and invasive lobular breast carcinoma (red arrow). All magnifications ×400, except A ×200.*

limited sample study reported a tenfold reduction in *Kiss-1* mRNA expression in breast cancer brain metastases compared to primary tumors (28). In the present study, paraffin-embedded tissues blocks obtained from 43 women diagnosed with primary breast carcinomas and 11 women suffering from fibrocystic disease, which served as controls, were submitted for immunohistochemistry. Our results demonstrate that Kiss-1 and GPR54 protein expression is increased in cancer tissues compared to non-malignant mammary specimens. Kiss-1 expression was also significantly and positively associated with GPR54 expression. The above results are in agreement with previous studies conducted by Martin *et al*. (43) and Marot *et al*. (44) which showed that Kiss-1 mRNA and protein levels are higher in breast cancer tumors compared to normal mammary parenchyma. On the contrary, Pentheroudakis *et al*. reported that in a cohort of 48 tumors only half (52%) were immunostained for kisspeptin (40% weakly and 12% moderately), in contrast to the strong universal staining observed in normal tissues (47). Increased Kiss1 expression has been correlated with advanced tumor grade (43, 44) and nodal positivity (43) in prior reports. In addition, higher Kiss-1 expression has been reporter in lobular compared to ductal carcinomas (43).

In the present study, Kiss-1 and GPR54 protein expression did not correlate with tumor grade, axillary nodal involvement or histological classification. However the sample size of our cohort may not have been large enough to allow for definitive conclusions. Hormonal receptor status though has been reported to correlate with Kiss-1 expression levels, with Era positive tumors expressing sevenfold lower Kiss-1 protein levels compared to Era-negative tumors (44). However, we did not find any significant correlations between Kiss-1 and ER, PR, cerb2, p53 or Ki67, while GPR54 expression was significantly and positively

associated only with cerb2 expression. In conclusion, the present study demonstrated that both Kiss-1 and GPR54 expression at the protein level was significantly higher in breast cancer compared to non-malignant breast tissues. These results further support the role of the Kiss-1/GPR54 system in breast cancer biology. Our evolving understanding for the design of effective therapeutic strategies aimed at modulating the Kiss-1/GPR54 pathway.

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