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Immunohistochemical study of KiSS1 and KiSS1R expression in human primary breast cancer: Association with breast cancer receptor status, proliferation markers and clinicopathological features

Katarzyna Jarzabek[1], Mariusz Koda[2], Leszek Kozlowski[3], Robert Milewski[4] and Slawomir Wolczynski[1]

[1]Department of Reproduction and Gynecological Endocrinology, Medical University of Bialystok, Poland, [2]Department of General Pathomorphology, Medical University of Bialystok, Poland, [3]Department of Surgical Oncology, Internal Affairs Hospital, Bialystok, Poland and [4]Department of Statistics and Medical Informatics, Medical University of Bialystok, Poland

Summary. Recent studies have raised doubts about the protective role of KiSS1/KiSS1R in breast malignancy progression. However, the role of the KiSS1/KiSS1R system in primary breast cancer remains largely unknown. The aim of the present study was to characterize the biology and invasiveness potential of primary breast cancer through evaluation of KiSS1/KiSS1R protein expression and cellular localization with regard to lymph node metastasis status, receptor status (ERs, PR and HER-2/neu), and expression of aromatase, MMP-9, Ki-67 and Cyclin D1 in primary invasive breast cancer tissues.

We showed increased protein expression of both KiSS1/KiSS1R and MMP-9 in the cancerous tissue compared with noncancerous tissue adjacent to the breast tumour. In the studied group of breast cancer samples, we observed a positive correlation between KiSS1 and MMP-9. We also showed a positive correlation between KiSS1R and aromatase expression in all studied breast cancers. We did not notice any associations between the KiSS1/KiSS1R system and cell cycle regulators. KiSS1/KiSS1R did not correlate either with Cyclin D1 and Ki-67 or with receptor status. However, we showed higher levels of KiSS1R expression in ERα-negative cases than in ERα-positive cases in patients with lymph node metastasis. Present data do not confirm the protective role of KiSS1/KiSS1R in breast cancer progression, but our results do support the hypothesis that the KiSS1/KiSS1R system is activated even in primary breast cancer and sustained during invasion to local lymph nodes.

Key words: KiSS1/KiSS1R, MMP-9, Primary breast cancer

Offprint requests to: Katarzyna Jarzabek, PhD., Department of Reproduction and Gynecological Endocrinology, Medical University of Bialystok, Sklodowskiej 24A, 15-276 Bialystok, Poland. e-mail: kasia.jarzabek@gmail.com

Introduction

Breast cancer is one of the major causes of cancer death in women. Despite its large molecular heterogeneity, the genesis and malignant progression of breast cancer are tightly connected to oestrogens and their receptors and dependent pathways. It is well known that as the malignant potential of breast cancer cells increases, the expression of genes involved in cell cycle control, tumour invasion and migratory properties is also altered. Thus, the identification of metastasis suppressor genes or genes promoting metastasis in the context of anticancer therapy is now one of the most important challenges in cancer research.

KiSS1, first known as a human metastasis suppressor gene, is an endogenous ligand for KiSS1R (known as GPR54), a G protein-coupled receptor (Kotani et al., 2001). The antimetastatic potential of KiSS1 has been described for numerous malignancies, including melanoma (Lee et al., 1996; Lee and Welch, 1997a,b; Shirasaki et al., 2001), lung (Zohrabian et al., 2007), thyroid (Ringel et al., 2002; Stathatos et al., 2005), ovarian (Hata et al., 2007), bladder (Cebrian et

al., 2011), gastric (Dhar et al., 2004; Guan-Zhen et al., 2007), pancreatic (Masui et al., 2004) and pituitary (Martínez-Fuentes et al., 2011) cancers.

In addition to its antimetastatic potential, KiSS1 has been discovered to play a pivotal role in other human physiological processes. The KiSS1/KiSS1R system is involved in placentation and trophoblast invasion (Janneau et al., 2002; Hiden et al., 2007) and leptin feedback (Smith et al., 2006; Crown et al., 2007; Quennell et al., 2011). KiSS1 is an essential factor in regulating the neuroendocrine reproductive axis during pubertal maturation (Thompson et al., 2004), and it plays an important role in LH/FSH secretion regulation in adulthood, as well (Navarro et al., 2005a,b). Indeed, mutations described in the *KiSS1* and *KiSS1R* genes have been connected with hypogonadism in humans (de Roux et al., 2003; Seminara et al., 2003) (the role of the KiSS1/KiSS1R system is summarized in Fig. 1). It has been proven that estradiol can up- or downregulate *KiSS1* gene expression in the hypothalamus. This regulation has been found to be ERα-dependent through Sp proteins whose sequences were found in the promoter region of the *KiSS1* gene (Li et al., 2007).

The role of the KiSS1/KiSS1R system in breast cancer pathophysiology in the context of oestrogen is still difficult to determine. Previous studies have suggested that KiSS1/KiSS1R signalling in breast cancer might play a metastatic suppressor role like that first described for melanoma. A variety of *in vitro* cell culture models have demonstrated that KiSS1R, activated by Kisspeptin, inhibits cell motility, proliferation, invasion and metastasis (Lee and Welch, 1997a,b; Martin et al., 2005; Olbrich et al., 2010). However, recent *in vitro* studies have raised doubts about the protective role of KiSS1/KiSS1R in breast malignancy. Heng et al. (2013) showed that activation of G protein-coupled receptors can facilitate metastasis through the degradation of the extracellular matrix. Additionally, Zajac et al. (2011) showed that Kisspeptin 10 signalling via KiSS1R stimulates MMP-9 secretion in MDA-MD-231 breast cancer cells depending on the presence of epidermal growth factor receptors (EGFR).

The aim of this study was to characterize the biology and invasiveness potential of primary breast cancer. To address this question, we applied immunohistochemistry (IHC) methods to primary invasive breast cancer tissues to define KiSS1 and KiSS1R protein levels and cellular localization in relation to lymph node metastasis status and MMP-9 expression; receptor status (oestrogen receptors alpha and beta [ERα and ERβ], progesterone receptor [PR] and HER-2/neu); aromatase expression (as an indicator of the oestrogen source in the breast tumour); and two cell cycle regulators, Cyclin D1 and Ki-67.

Materials and methods

Patients and samples

Breast cancer samples were obtained from 54 women after surgical treatment. Samples were fixed in 10% (v/v) buffered formaldehyde solution for 48 h and then embedded in paraffin blocks at 56°C according to standard procedures. Standard histopathological parameters were determined by two independent pathologists.

Tumour samples were cut into 5 μm thick sections and stained with hematoxylin-eosin. Histopathological examination was based on the WHO and pTN classification of breast tumours (Tavassoli and Devilee, 2003). Histopathological grading (G) was performed according to the Bloom and Richardson system (Bloom and Richardson, 1957). Patients had not received any preoperative chemo- or hormonotherapy. The age of patients ranged from 48 to 88 years (mean 59 years). The ethical committee of Medical University of Bialystok approved the protocol of this study.

For immunohistochemical studies we selected 2 representative sections from each case. Ten markers were investigated using IHC study: KiSS1, KiSS1R, ERα, ERβ, PR, HER-2/neu, Cyclin D1, MMP-9, aromatase and Ki-67. KiSS1 and KiSS1R were assessed with a monoclonal anti-KiSS1 antibody (M05) clone 1F7 (Abnova) and polyclonal anti-KiSS1R antibody SP 4237P (Acris, Germany) respectively, both at dilution 1:200. ERα was detected with a mouse monoclonal antibody (Dako, Denmark) at dilution 1:100, ERβ was detected with a monoclonal Ab EMR02 (NCL-ER-beta, Novocastra) at dilution 1:100, PR was assessed using monoclonal Ab (Dako, Denmark) at dilution 1:50, Ki-67 and Cyclin D1 were assessed using the monoclonal mouse Abs MIB-1 (Dako, Denmark) at 1:100 dilution. MMP-9 was assessed using monoclonal mouse Ab (2C3, Santa Cruz Biotechnology) at dilution 1:100. Aromatase expression was assessed using a rabbit polyclonal Ab R-10-2 against cytochrome P450-aromatase at dilution 1:800 (a generous gift from Dr. Yoshio Osawa, Hauptman-Woodward Medical Research Institute, Buffalo, NY, USA).

The sections were deparaffinized in xylenes and hydrated through graded alcohols. Antigen unmasking was performed using heat treatment in a microwave oven at 750 W for 6 minutes in a container with 10 mM sodium citrate buffer, pH 6.0. The sections were allowed to cool in the buffer at room temperature for 30 minutes and were rinsed in deionized H_20 three times for 2 minutes each. The endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 20 minutes. After rinsing in PBS, the sections were incubated for 1 hour with a proper 1.5% normal blocking serum in PBS. The blocking reagent was removed and then the sections were incubated with ERα, PR, Ki-67 and Cyclin D1 antibodies for 1 hour at room temperature or with KiSS1, KiSS1R, ERβ and aromatase antibodies at 4°C overnight using staining chamber (The Binding Site, United Kingdom). Primary antibodies were diluted in PBS with 1.5% normal blocking serum. The studies for assessed markers were performed with EnVision system (Dako, Denmark) to reveal Ab-antigen reactions. Staining was routinely developed using 3,3'-

diaminobenzidine as a chromogen (Dako, Denmark). The sections were counterstained with hematoxylin.

Immunoreactivities were scored in light microscopy in 10 different fields under magnification of 200x and the mean percentage of tumour cells which showed positive staining was assessed. In discriminating protein-positive from negative tumours, the cut-off was 10% malignant cells that expressed the investigated marker from all the cancer cells of the microscopic slide.

The expression of ERs, PR, Cyclin D1, KiSS1, KiSS1R and Ki-67 was graded on a three-tiered system: 0 (defined as negative cases) less than 10% positive cells; 1+ with immunoreactivity exceeding from 10-50% positive cancer cells; 2+ with over 50% positive cells. The expression of MMP-9 was graded on a four-tiered system: 0 (defined as negative cases) less than 10% positive cells (thus this was in reality very weakly positive result); 1+ with immunoreactivity exceeding from 10-50% positive cancer cells (moderately positive); 2+ with over 50% positive cells; 3+ over 50% positive cells with strong reaction. The expression of aromatase was graded on a five-tiered system: 0 (defined as negative cases) less than 10% positive cells (thus this was in reality a very weakly positive result); 1+ with immunoreactivity exceeding from 10-50% of positive cancer cells (moderately positive); 2+ with over 50% of positive cells; 3+ 50-75% of positive cancer cells with a strong reaction; 4+ over 75% of positive cancer cells with a strong reaction.

For evaluation of HER-2/neu status, 2 representative sections from each case of breast cancer tissues were selected. The procedure according to Ventena Medical System CONFIRM was applied as a routine diagnostic tool (Ventena Medical System, USA). The HER-2/neu staining was carrying out under the conditions recommended by the manufacturer using Pathway HER-2/neu (4B5).

Table 1. Clinicopathological characteristics of 54 primary breast canceers.

Parameter	No. of cases
Tumour stage	
pT1	10
pT2	44
Nodal status	
pN0	25
pN1	15
pN2	10
pN3	4
Carcinoma ductale invasivum (Invasive carcinoma of no special type)	
G2	28
G3	17
Carcinoma lobbulare invasivum	9
Age (years)	
≤ 60	31
>60	23

Statistical analysis

Mean values ± standard deviation (SD) and median were calculated. The normal distribution was verified using the Kolmogorov-Smirnov test with the Lilliefors correction. The Pearson's chi-squared test was used. To compare the differences between the two groups, the Mann-Whitney U test was used. The Spearman's correlation coefficients were also estimated. The analysis was performed using the statistical software package Statistica 10.0 (StatSoft) with $P<0.05$ indicating a statistically significant difference.

Results

Clinicopathological features of the studied breast cancer cohort

The age of patients (n=54) ranged from 48 to 88 years (mean 59 years). Tumour stage pT, nodal status (pN), Bloom and Richardson grading (G) and age range of breast cancer patients are presented in Table 1. Breast cancer characteristics by receptor status (ERα, ERβ, PR and HER-2/neu) and Ki-67 proliferation marker are shown in Table 2.

Expression profile and localization of ERs, PR, KiSS1, KiSS1R, MMP-9, Cyclin D1 and aromatase

Antigen expressions were analysed by IHC in all the primary tumour samples. In some IHC stainings, we obtained no interpretable data. The total number of analysed cancer tissues was as follows: KiSS1, n=53; KiSS1R, n=51; aromatase, n=50; Cyclin D1, n=53; and MMP-9, n=50. Table 3 shows the immunoreactivity of KiSS1, KiSS1R, MMP-9, Cyclin D1 and aromatase (graded on a three-tiered system for KiSS1, KiSS1R and Cyclin D1; four-tiered for MMP-9; and five-tiered for aromatase).

Table 4 shows results concerning KiSS1, KiSS1R, MMP-9 and Cyclin D1 expression depending on the lymph node status and presence of ERα and HER-2/neu.

Among the 54 analysed cases, 37/54 (68.5%) were ERα-positive, while 35/54 (65%) were both ERα- and PR-positive. HER-2/neu overexpression was found in

Table 2. Characteristics of studied breast tumours with regard to receptor status and proliferation marker expression (Ki-67).

Studied marker	Immunostaining (n/%)			
	0	1+	2+	3+
HER2/neu/n=54	39/72%	2/4%	2/4%	11/20%
ERα/n=54	17/31.5%	12/22.2%	25/46.3%	-
ERβ/n=54	12/22.2%	15/27.8%	27/50%	-
PR/n=54	19/35.2%	11/20.4%	24/44.4%	-
Ki-67/n=54	8/14.8%	36/66.7%	10/18.5%	-

11/54 (20%) cases, and 39/54 (72%) cases were HER-2/neu-negative. Additionally, MMP-9, KiSS1 and KiSS1R were assessed in noncancerous tissue adjacent to breast cancer tissue. We found focal positive cytoplasmic immunoreactivity for MMP-9 in 19/38 of benign mammary lesions adjacent to breast cancer. For KiSS1 and KiSS1R, weak expression was observed in 25/51 and 19/40 of noncancerous tissues adjacent to breast cancer, respectively.

KiSS1 was detected in cytoplasm both in epithelial breast cancer cells (Fig. 2A) and in matched tissues (Fig. 2B). We also observed a positive KiSS1 reaction in a few inflammatory cells. Of the studied cancerous tissues, 26% (14/53) were KiSS1 negative, compared with 51% (26/50) of matched tissues. The level of KiSS1R protein was also significantly higher in breast cancer tissues than in the matched tissues: KiSS1R was overexpressed in 45% (23/51) of analysed breast cancer tissues, while 52.5% (21/40) of matched tissues were KiSS1R negative. KiSS1R was localized predominantly in the cytoplasm in both cancerous cells and matched tissue (Fig. 2D). Interestingly, 7 breast cancer cases (of 51 studied samples) showed membrane KiSS1R immunoreactivity (Fig. 2C). Additionally, immunostaining for KiSS1R was found in some stromal (mainly inflammatory) cells. We observed MMP-9 expression mainly in the cytoplasm of epithelial breast cancer cells (Fig. 2E) in 74% (37/50) of breast cancer tissues; 46% (23/50) of the same showed MMP-9 overexpression. Only a few inflammatory cells showed a weak positive MMP-9 expression. We observed no MMP-9 immunoreactivity in 50% (19/38) of matched tissues. Observed positive MMP-9 immunoreactivity in matched tissues was notably weak (Fig. 2F). In the studied breast cancer samples, 57% (30/53) showed a very strong Cyclin D1 immunoreaction in the nuclei of cancerous cells (Fig. 2G); however, in matched tissue, we observed only focal Cyclin D1 expression (Fig. 2H). Half (25/50) of the studied breast cancer samples showed very strong (+3 and +4) aromatase immunoreactivity. Aromatase expression was detected mainly in the cytoplasm of epithelial cancer cells, and only weak immunostaining

Table 3. Studied marker expression and distribution with regard to immunostaining intensity.

Studied marker	Immunostaining (n / %)				
	0	1+	2+	3+	4+
KiSS1/n=53	14/26.4%	25/47.2%	14/26.4%	-	-
KiSS1R/n=51	14/27.5%	14/27.5%	23/45%	-	-
Cyclin D1/n=53	8/15%	15/28%	30/57%	-	-
MMP-9/n=50	13/26%	14/28%	11/22%	12/24%	-
Aromatase/n=50	2/4%	10/20%	13/26%	12/24%	13/26%

Table 4. KiSS1, KiSS1R, MMP-9 and Cyclin D1 expression in primary breast tumour in relation to lymph node metastasis, ERα and HER-2/neu status.

		KiSS1 (n=53)				KiSS1R (n=51)	
	n / analysed tumors	Negative	Mild	Severe	n / analysed tumors	Negative	Mild	Severe
Lymph node metastasis								
Negative	24/53	5	13	6	24/51	4	7	13
Positive	29/53	9	12	8	27/51	10	7	10
ERα								
Negative	16/53	4	8	4	16/51	3	4	9
Positive	37/53	10	17	10	35/51	11	10	14
HER2/neu								
Negative	39/53	10	18	11	36/51	10	10	16
Positive	14/53	4	7	3	15/51	4	4	7
		MMP-9 (n=50)				CyclinD1 (n=53)	
	n / analysed tumors	Negative	Mild	Severe	n / analysed tumors	Negative	Mild	Severe
Lymph node metastasis								
Negative	23/50	5	8	10	25/53	5	9	11
Positive	27/50	8	6	13	28/53	3	6	19
ERα								
Negative	15/50	5	5	5	17/53	6	5	6
Positive	35/50	8	9	18	36/53	2	10	24
HER2/neu								
Negative	37/50	8	12	17	38/53	7	13	18
Positive	13/50	5	2	6	15/53	1	2	12

KiSS1, KiSS1R and Cyclin D1 were graded on a three-tiered system. MMP-9 was graded on a four-tiered system: +2 and +3 MMP-9 grades were got together in this table and present as severe immunoreactivity.

was found in part of normal as well as in stromal cells.

Correlations among ERα, ERβ, KiSS1, KiSS1R, MMP-9, Cyclin D1 and aromatase

KiSS1 protein expression correlated positively with expression of both KiSS1R and MMP-9 (P<0.03, r=0.3, and P<0.0001, r=0.77, respectively). Statistically significant positive correlations were also found between KiSS1R and aromatase expression (P<0.002, r=0.44) and between ERα and Cyclin D1 expression (P<0.005, r=0.39). Negative correlations were found between ERα expression and Ki-67 status (P<0.00001, r=-0.56) and between PR and Ki-67 (P<0.00008, r=-0.51). Cyclin D1 was negatively associated with Ki-67 status (P<0.005, r=-0.38).

Correlations of KiSS1, KiSS1R, MMP-9, Cyclin D1, ERα, ERβ and aromatase expression with selected clinical and pathological features

The quantitative relationships among mRNA of *KiSS1, KiSS1R, Cyclin D1*, aromatase, ERs and PR in the same set of breast cancer samples has already been published (Jarząbek et al., 2012). The coefficient factors between mRNA and protein levels for the studied factors were as follows: for ERα, P<0.000001; for ERβ, P<0.125; for KiSS1, P<0.3; for KiSS1R, P<0.5; for Cyclin D1, P<0.0027; for aromatase, P<0.56.

No significant difference was seen between tumour size (pT) and the expression of studied proteins. Moreover, no significant correlation was observed between lymph node metastasis status and the expression of studied antigens. However, in a group of patients with lymph node metastases, the KiSS1R protein level was increased among ERα-negative cases (P<0.05). These results diverge from data analysed for KiSS1R mRNA expression, where an elevated transcription level was observed in the ERα-positive group with lymph node metastasis (P<0.005). In the ERα-positive group without lymph node metastasis, we observed overexpression of Cyclin D1. More than 75% of studied breast cancer tissues in this group showed very strong Cyclin D1 nuclear immunoreaction (P<0.001). Taking all analysed cancerous tissues into consideration, the ERα level was significantly higher in HER-2/neu-negative tumours (P<0.05). At the protein level, we did not confirm data obtained from our RT-qPCR study that showed higher KiSS1R levels in HER-2/neu-negative tumours (Jarząbek et al., 2012).

Discussion

The role established for the KiSS1/KiSS1R system in breast cancer biology is more complicated than initially thought. The statement that the KiSS1/KiSS1R

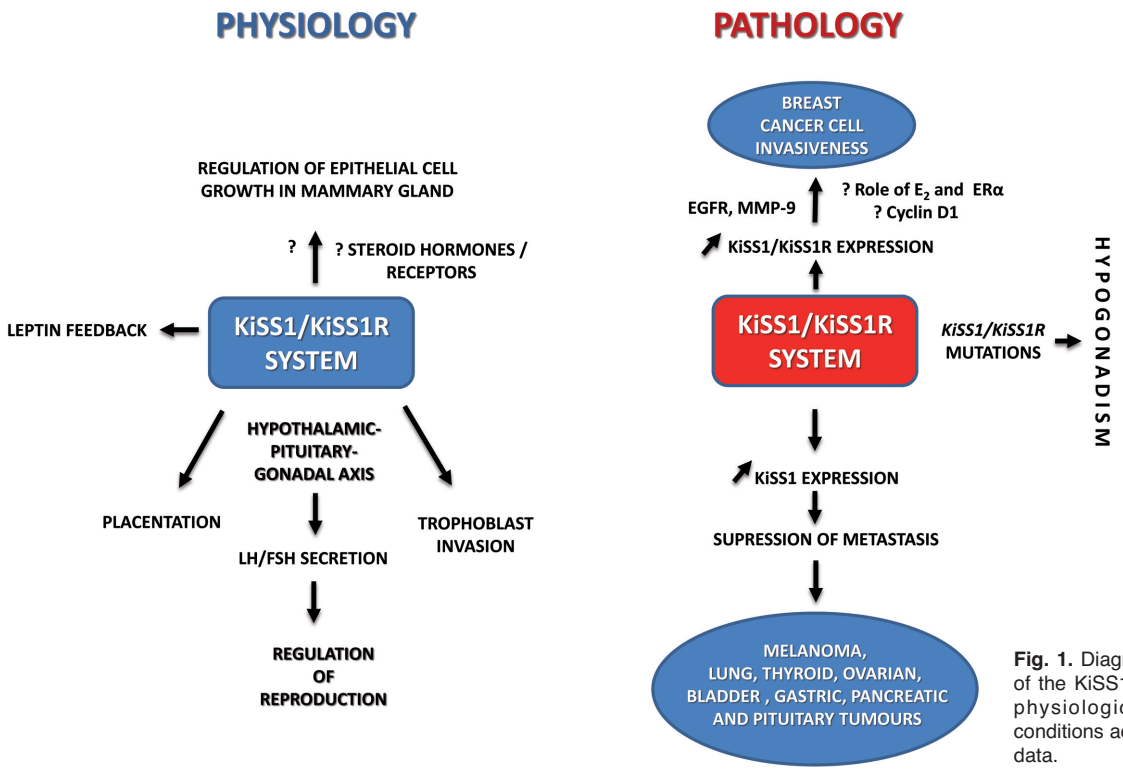

Fig. 1. Diagram summarizing a role of the KiSS1/KiSS1R system in the physiological and pathological conditions according to the literature data.

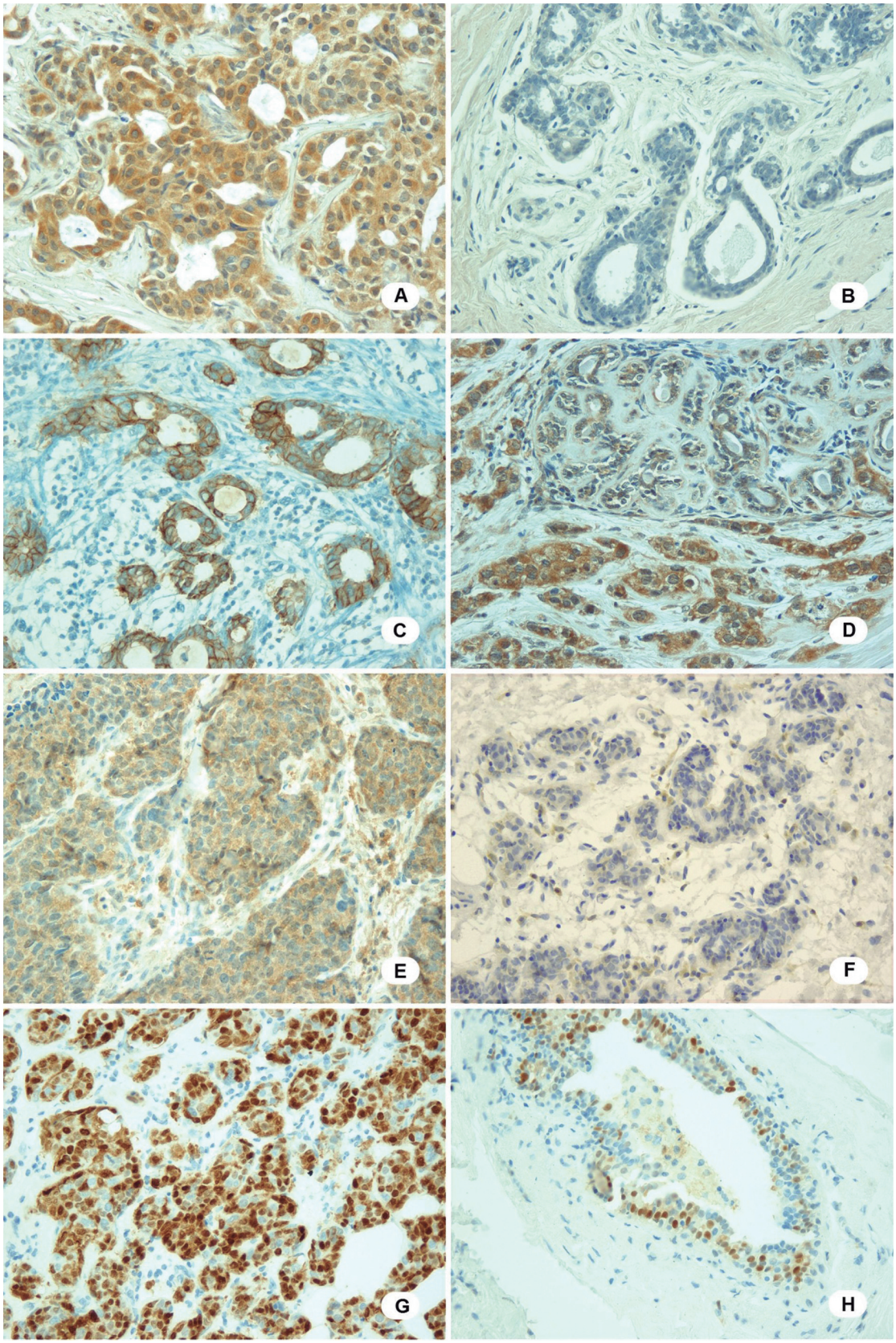

Fig. 2. A-H. Expression of KiSS1, KiSS1R, MMP-9 and Cyclin D1 in human primary breast cancer and tissue adjacent to tumour. Strong cytoplasmic immunostaining of KiSS1 in primary breast cancer cells **(A)** with focal cytoplasmic immunoreactivity in matched tissue **(B)**. Strong membrane KiSS1R localization in breast cancer cells **(C)**. Mainly cytoplasmic immunostaining of KiSS1R in cancerous cells with focal cytoplasmic immunoreactivity in adjacent noncancerous tissue **(D)**. Cytoplasmic localization of MMP-9 in cancerous cells **(E)** with very weak cytoplasmic immunostaining in adjacent tissue **(F)**. Strong nuclear expression of Cyclin D1 in breast cancer cells **(G)** with focal nuclear Cyclin D1 immunoreactivity in matched noncancerous tissue **(H)**. Original magnification: x 200

signalling system may be a universal originator of metastasis suppression capable of inhibiting metastases to other human body sites regardless of the cancer origin does not apply to all types of cancers and, especially for breast cancer, should be verified.

In this study, we sought to characterize early events potentially responsible for the metastatic process in breast cancer by evaluating KiSS1/KiSS1R protein expression in primary invasive breast tumours, as well as in benign mammary lesions adjacent to the studied breast cancer. Contrary to work conducted by Ulasov et al. (2012) in which no significant difference was observed between KiSS1 expression in primary breast cancer and non-neoplastic breast parenchyma, we showed elevated KiSS1 and KiSS1R expression levels in primary breast cancer in comparison to matched tissue. Extremely weak or undetectable KiSS1/KiSS1R immunohistochemical reactions were observed in breast tissues adjacent to breast cancer. KiSS1 and KiSS1R, were both localized in the cytoplasm of epithelial cancerous cells; however, we also observed membrane localization of KiSS1R in 7 of 54 of studied breast tumours. Positive KiSS1/KiSS1R reactions observed in a few leukocytes and macrophages suggest involvement in an inflammatory process. Our results may imply an increase in KiSS1/KiSS1R expression during breast carcinogenesis, and this local KiSS1/KiSS1R overexpression might play a significant role in the initiation of invasiveness potential in the human breast. Studies based on xenograft murine models and studies using human breast cancer cell lines have both revealed antimetastatic potential of overexpressed KiSS1 (Lee and Welch, 1997a,b; Nash et al., 2007); however, conclusions from these results should be interpreted with careful consideration. These models have some limitations in terms of understanding the role of KiSS1/KiSS1R in initiation and early events of breast tumour formation and progression.

The role of KiSS1/KiSS1R in regulating breast cancer metastasis has been recapitulated using a murine model of *Kiss1r* gene knockout in which breast cancer induction was performed using murine mammary virus-polyoma virus middle T antigen (MMTV-PyMT) (Cho et al., 2011). In this study, the authors showed that *Kiss1r* haploinsufficiency delayed virus-induced breast tumour initiation, latency, growth and metastasis. Moreover, a reduced expression of Kiss1r inhibited tumour growth *in vivo* (Cho et al., 2011).

Marot et al. concluded that *KiSS1* and *KiSS1R* are oestrogen-regulated genes and that their expression level in breast cancer tissue must be analysed together with ER status (Marot et al., 2007). It has been postulated that in postmenopausal women, oestrogen is produced by intratumoural, locally expressed aromatase in both cancerous breast tissue and surrounding stromal cells, and this in turn may lead to the oestrogen-sensitive proliferation of epithelial breast cancer cells. In our study, we showed a positive correlation between aromatase and KiSS1R expression. The immunoreactivity of aromatase also showed a trend toward positive correlation with KiSS1 (data not shown). Although previous data from our RT-qPCR study showed higher *KiSS1* and *KiSS1R* mRNA levels in ER-positive than ER-negative breast cancers, we showed no associations at a protein level between ERs and KiSS1/KiSS1R protein expression. Interestingly, when we analysed KiSS1/KiSS1R protein expression levels in relation to lymph node metastasis status, we noticed an elevated KiSS1R protein level in a group of patients with lymph node metastases among ERα-negative cases. Higher KiSS1R expression in ERα-negative versus ERα-positive cancers with lymph node metastasis may favour the hypothesis that KiSS1R can induce invasiveness in ER-negative mammary epithelial cancer cells. Cvetkovic et al. (2013) also demonstrated that KiSS1R expression in noninvasive ER-negative MCF10A cells induces an epithelial-mesenchymal transition-like event through upregulation of mesenchymal markers, such as N-cadherin and Snail/Slug. Moreover, Zajac et al. (2011), using *in vitro* studies, established pro-migratory and pro-invasive roles for KiSS1/KiSS1R signalling in breast cancer. ERα and oestrogen were proposed to be negative regulators of KiSS1R in breast cancer cells. Our data only partially confirm this statement. On the one hand, KiSS1R expression is higher in ERα-negative cases among patients with lymph node metastasis; on the other hand, we show a positive correlation between KiSS1R and aromatase expression in all studied breast cancers. It is known that dynamic changes in the extracellular matrix during carcinogenesis are responsible for acquisition of both malignant potential and the facility of breast cancer cells to invade the basal membrane and metastasize. Despite reported findings of high MMP-9 expression in high-grade tumours (Sullu et al., 2011), we did not observe any associations between MMP-9 expression and clinicopathological features. However, MMP-9 has been shown elsewhere to be involved in the invasiveness of breast cancer cells. Zajac et al. (2011) showed that Kisspeptin-10 participates in breast cancer cells' invasiveness through MMP-9 production depending on EGFR. In our study, we showed a very strong positive correlation between KiSS1 and MMP-9 protein expression, indicating a possible common mechanism of regulation in breast cancer. However, we did not notice any correlation between HER-2/neu, which belongs to the EGFR family, and MMP-9 in studied breast cancer patients.

Although our previous mRNA analysis revealed a lower *KiSS1R* expression level in HER-2/neu-positive breast tumours than in HER-2/neu-negative tumours (Jarząbek et al., 2012), the present data based on protein expression did not confirm these results. The discrepancy in the data concerning KiSS1R obtained at mRNA and protein levels may indicate that the RT-qPCR method is insufficient for the characterization of studied markers in such heterogenic samples as breast cancer tissues.

Interestingly, we did not observe any correlations between the KiSS1/KiSS1R system and cell cycle mediators Cyclin D1 and Ki-67, proteins which are altered during the cell cycle progression in breast cancer patients. Cyclin D1 was positively correlated with ERα; however, intensity of Ki-67 nuclear expression decreased with high ERα expression in cancerous cells. We noticed an irreversible association between immunohistochemically detected expression of Cyclin D1 and Ki-67. Moreover, we showed that more than 75% of studied breast tumours overexpressed Cyclin D1 in ERα-positive tumours without lymph node metastasis. The coexistence of ERα and Cyclin D1 in breast cancer has been explained as the consequence of E_2 regulation of Cyclin D1 or as the result of functional crosstalk between ERα coactivators and Cyclin D1 that activate oestrogen response element (Moghadam et al., 2011). Our results suggest that in the studied breast cancers, ERα may activate a gene responsible for proliferating activity early in the cell cycle.

In summary, a comparison of primary invasive breast cancer tissues with adjacent noncancerous tissues showed increased protein expression of both KiSS1/KiSS1R and MMP-9 in the cancerous tissues. It remains to be determined whether oestrogens produced locally by aromatase or ERα itself may enhance KiSS1/KiSS1R signalling in primary breast cancer. It is interesting that in the studied group of breast cancer samples, 70% of which were ERα-positive, we observed a positive correlation between KiSS1 and MMP-9. Present data do not confirm the protective role of KiSS1/KiSS1R in breast cancer progression; however, our results support the hypothesis that the KiSS1/KiSS1R system is activated even in primary breast cancer and is sustained during invasion to local lymph nodes.

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Conflict of interest statement. The authors declare no conflict of interest.

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