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ASSOCIATION BETWEEN THE EXPRESSION OF KISS1, KISS1R AND MMP-9 IN INVASIVE BREAST CARCINOMAS

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Abstract

Metastases are the leading cause of fatal outcome in patients with breast cancer, therefore different proteins related to metastasis are widely studied for the potential to control the spread of cancer cells. It has been shown in various types of cancers that the anti-metastatic role of the KISS1/KISS1R system has been performed by suppressing metalloproteinase 9 (MMP-9) expression, but there is evidence that the relationship between KISS1/KISS1R signalling and MMP-9 in breast cancer seems to be different. The aim of the current study was to assess the association between protein expression of KISS1, KISS1R and MMP-9 using software-based measurement of staining intensity and the possible link to tumour size, grade and receptor status in invasive breast carcinomas. Standard histopathological parameters were determined by an experienced pathologist and immunohistochemical staining for KISS1, KISS1R and MMP-9 was performed on tissue samples from 54 patients. Evaluation of staining intensity was performed using ImageJ software on RGB images by two operators. Results showed significant strong positive correlation between KISS1, KISS1R and MMP-9 and strong expression of studied proteins in tumour associated stromal cells. No association was found between the expression of the studied proteins and size, receptor status or grade. Higher expression of KISS1R, however, was observed in HER2-negative compared to HER2-positive

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carcinomas, which might indicate an alternative pathway for stimulating proliferation of tumour cells when HER2 expression is low.

Key words: KISS1, KISS1R, MMP-9, metastasis promoter, invasive breast carcinomas

Introduction. Breast cancer is the most common malignancy in women with the vast majority of cancer-related deaths resulting from systemic dissemination of tumour cells, rather than primary tumour growth. Therefore, recent research has mostly been focused on identifying metastasis suppressor genes. One such gene is KISS1 that has first been discovered for its ability to suppress metastases in human melanoma cell lines [1]. Subsequently it became clear that the gene encodes a propeptide that is unstable and biologically inactive but cleaves into four biologically active peptides that differ in the number of amino acids: kisspeptin-10, 13, 14, and 54. All of them have the same C-terminal region by which they bind to and fully activate the G-protein coupled receptor 54 (GPR54), recently referred to as KISS1 receptor (KISS1R). Because of these structural similarities, all four biologically active peptides are collectively referred to as "kisspeptins" and are commonly designated as KISS1, for being the products of the KiSS1 gene.

The anti-metastatic properties of KISS1/KISS1R signalling have been demonstrated in many types of cancer, including melanoma, thyroid, ovarian, bladder, gastric, oesophageal, pancreatic, lung and pituitary cancers. For several of these cancer types it has been revealed that metastasis suppression is achieved by KISS1/KISS1R-dependent suppression of matrix metalloproteinase 9 (MMP-9) activity and subsequent inhibition of cancer cell migration and invasion [2]. Further studies on breast cancer, however, revealed that KISS1/KISS1R signalling promotes invasion and metastasis [3–6].

Matrix metalloproteinases are important regulators of tumour microenvironment which are produced both by tumour cells and stromal cells in the tumour microenvironment. MMP-9 is considered important for degradation of the basement membrane and extracellular matrix during cancer invasion and is therefore strongly associated with tumour progression and metastasis.

The aim of the current study was to assess the association between protein expression of KISS1, KISS1R and MMP-9 using software-based measurement of staining intensity and the possible link to tumour size, grade and receptor status in invasive breast carcinomas.

Materials and methods. ***Tissue samples.*** Breast cancer samples were collected from 54 female patients following surgical treatment. Samples were fixed in 10% (v/v) neutral buffered formalin and embedded in paraffin. Histopathological determination of tumour size, type, grade, and receptor status was performed.

Immunohistochemical staining. Immunostaining using rabbit polyclonal antibodies against KISS1 (at dilution 1:75), KISS1R (at dilution 1:100) and MMP-9 (at dilution 1:200) (Elabscience Biotechnology Inc., USA) was performed on

4 µm thick paraffin sections. After deparaffinization and rehydration, slides underwent heat-induced epitope retrieval in citric buffer pH 6.0 (ScyTek Laboratories Inc., USA) at 95 °C for 20 min. Endogenous peroxidase activity was blocked in 3% H_2O_2 for 10 min at room temperature. Subsequently, the sections were washed in TTBS (50 mM Tris HCl pH 7.6, 150 mM NaCl + 0.05% Tween 20) and incubated overnight at 4 °C with primary antibodies. Biotin-Streptavidin HRP detection system (ScyTek Laboratories Inc., USA) with DAB as chromogen was used. Slides were counterstained with haematoxylin, dehydrated and cover-slipped.

Evaluation of cytoplasm staining intensity using ImageJ. Intensity of staining was measured using ImageJ software and the resulting "reciprocal intensity" (RI) values were used to analyse the correlation between protein expression of KISS1, KISS1R and MMP-9.

For each sample, RGB images of three representative fields of vision with resolution 2576 × 1932 pixels saved as `.jpeg` were captured using Olympus BX51 microscope equipped with Olympus C5050Z camera at magnification 400×.

The evaluation of cytoplasm staining intensity of tumour cells using ImageJ was performed on the RGB images by two operators.

The first step of analysis included colour deconvolution (H DAB) and intensity of cytoplasm staining was measured in deconvoluted DAB image. The average staining intensity for each sample was calculated as the mean value of the measured "mean grey value" for 50 ROIs (regions of interest) selected manually from all 3 images (Fig. 1). Intensity values in ImageJ range from 0 to 255 with the lower values corresponding to higher intensities which would complicate data analysis and this was avoided by calculating RI of the stained area by subtracting the grey value measured for each ROI from 255. The resulting value is directly proportional to the amount of chromogen present [7].

Receptor status assessment. Receptor status was assessed using the Allred scoring system for ER and PR and a four-tier scoring system (0, 1+, 2+,

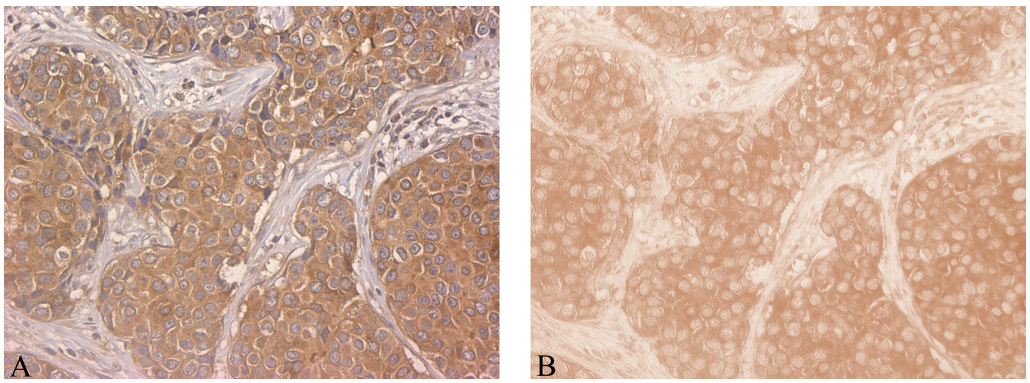


Fig. 1. Original RGB image (A) and the same image following deconvolution (B)

3+) for HER2. Scores of 0–2 were considered negative for ER and PR and scores of 3–8 were considered positive for ER and PR. For HER2, scores of 0 and 1+ were considered HER2-negative and scores of 3+ were considered HER2-positive. Scores of 2+ were considered equivocal and were resolved by retesting using DISH (dual in situ hybridization).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 software. The association between KISS1, KISS1R and MMP-9 was compared using Spearman's correlation coefficient. A p value of less than 0.05 was considered to indicate a statistically significant result.

Results. A total of 54 cases of invasive ductal carcinomas were studied. Patient age ranged from 27 to 85 (mean age 60.28 years).

Basic characteristics of the studied invasive breast carcinomas and the results from the assessment of receptor status are summarized in Table 1.

Immunohistochemistry revealed that all three studied proteins were mainly located in the cytoplasm of carcinoma cells. Stronger staining of immune cells, compared to tumour cells was observed in some of the cases (Fig. 2), but this was not taken into account when measuring staining intensity.

T a b l e 1

Basic characteristics of the studied invasive breast carcinomas ($n = 54$)

	n	%
Histological type		
Invasive ductal carcinoma	48	88.9
Invasive lobular carcinoma	5	9.3
Invasive micropapillary carcinoma	1	1.9
Size		
< 2 cm	15	27.8
2–5 cm	37	68.5
> 5 cm	2	3.7
Grade		
G1	2	3.7
G2	22	40.7
G3	30	55.6
ER status		
ER (−)	12	22.2
ER (+)	42	77.8
PR status		
PR (−)	21	38.9
PR (+)	33	61.1
HER2 status		
HER2 (−)	48	88.9
HER2(+)	6	11.1

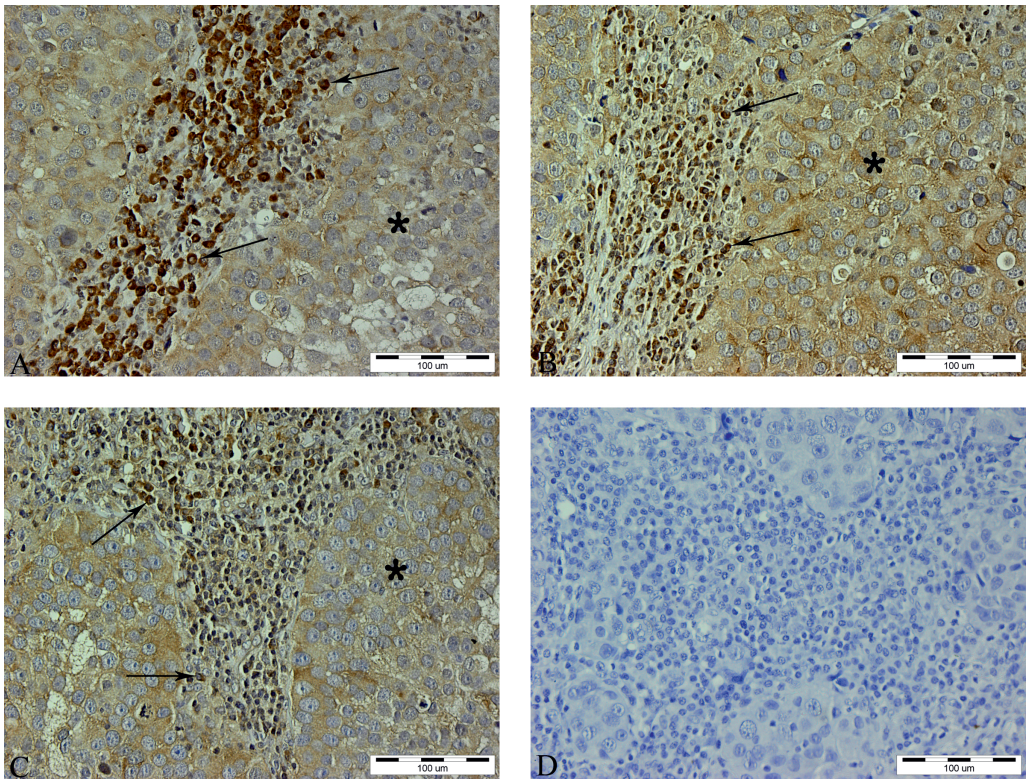


Fig. 2. Stronger staining of immune cells (arrows) compared to tumour cells (asterisk) for KISS1 (A), KISS1R (B) and MMP-9 (C); No primary antibody control (D)

The three studied proteins showed significant positive correlation between each other. KISS1 correlated better with KISS1R ($r = 0.66$, $p < 0.0001$) than with MMP-9 ($r = 0.59$, $p < 0.0001$). The strongest correlation was observed between KISS1R and MMP-9 ($r = 0.80$, $p < 0.0001$).

Calculated RI values were used to assess the association between the expression of KISS1, KISS1R and MMP-9 and tumour size, grade, and receptor status. No association was found between protein expression and tumour size, grade or receptor status for KISS1 and MMP-9, however, protein expression levels of KISS1R were significantly higher in HER2-negative tumours ($p = 0.0431$) (Fig. 3).

Discussion. Immunohistochemistry is a well-established method for detecting protein expression that is used in both clinical and research practice. Evaluation of staining intensity for clinical prognostic markers such as ER, PR and HER2 has been standardized, however, studying potential new markers is challenging, since assessment of staining intensity under the light microscope is known to be subjective. In such cases, software-based measurement of staining intensity might be more appropriate, since it provides a more objective and customizable alternative, especially when only a certain type of positive cells or structures in the tissue are intended for analysis.

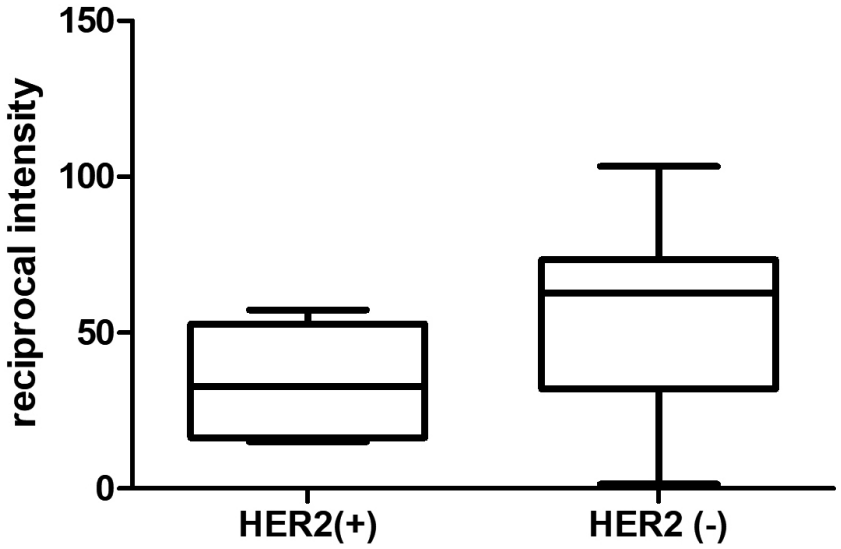


Fig. 3. Difference in protein expression of KISS1R between HER2-positive and HER2-negative tumours (Mann–Whitney U test)

By combining manual selection of ROI and software-based measurement we tried to overcome the limited accuracy and consistency of the manual scoring by pathologist and the unintentional biases in the assessment of staining intensity. Furthermore, the resulting numerical data facilitate statistical analysis.

Correlation between KISS1 and MMP-9 has been studied in different types of tumours and negative correlation has been reported in ovarian cancer [8], colorectal cancer [9], hepatocellular carcinoma [10], and osteosarcoma [11] which supports the anti-metastatic role of KISS1/KISS1R system in these types of cancer.

In accordance with the growing evidence for altered functioning of KISS1/KISS1R signalling in breast cancer, positive correlation between KISS1, KISS1R and MMP-9 is not surprising. Similar results were reported by Jarzabek et al. [12] who demonstrated positive correlation between KiSS1 and MMP-9. Though all three proteins were mainly located in the cytoplasm of tumour cells, strong positive staining of stromal cells was also observed, which suggests involvement of studied proteins in the host response against tumour cells.

In the present study we found higher protein levels of KISS1R in HER2-negative compared to HER2-positive tumours which supports the reported difference in KISS1R mRNA expression levels between HER2 positive and HER2 negative tumours and might indicate an alternative pathway for stimulating proliferation of tumour cells when HER2 expression is low [13].

Conclusion. Positive correlation in protein expression of KISS1, KISS1R and MMP-9 further supports the metastasis promoting role of KISS1/KISS1R signalling and emphasizes the importance of the interaction between KISS1/KISS1R

system and MMP-9 in the context of invasive breast carcinomas. Strong expression of studied proteins in stromal cells suggests involvement in the host response against tumour cells. Higher expression of KISS1R in HER 2-negative carcinomas might indicate an alternative pathway for stimulating proliferation of tumour cells when HER2 expression is low.

REFERENCES

[1] Lee J. H., D. R. Welch (1997) Identification of highly expressed genes in metastasis-suppressed chromosome 6/human malignant melanoma hybrid cells using subtractive hybridization and differential display, Int. J. Cancer., **71**, 1035–1044.

[2] Makri A., N. Pissimissis, P. Lembessis, C. Polychronakos, M. Koutsilieris (2008) The kisspeptin (KiSS-1)/GPR54 system in cancer biology, Cancer Treat. Rev., **34**, 682–692.

[3] Blake A., M. Dragan, R. G. Tirona, D. B. Hardy, M. Brackstone et al. (2017) G protein-coupled KISS1 receptor is overexpressed in triple negative breast cancer and promotes drug resistance, Sci. Rep., **7**, 46525, 1–17.

[4] Zajac M., J. Law, D. D. Cvetkovic, M. Pampillo, L. McColl et al. (2011) GPR54 (KISS1R) transactivates EGFR to promote breast cancer cell invasiveness, PLoS ONE, **6**(6), e21599.

[5] Marot D., I. Bieche, C. Aumas, S. Esselin, C. Bouquet et al. (2007) High tumoral levels of Kiss1 and G-protein-coupled receptor 54 expression are correlated with poor prognosis of estrogen receptor-positive breast tumors, Endocr. Relat. Cancer, **14**, 691–702.

[6] Martin T. A., G. Watkins, W. G. Jiang (2005) KiSS-1 expression in human breast cancer, Clin. Exp. Metastasis, **22**, 503–511.

[7] Nguyen D. H., T. Zhou, J. Shu, J. H. Mao (2013) Quantifying chromogen intensity in immunohistochemistry via reciprocal intensity, Cancer InCytes, **2**(1), 4 pp.

[8] Gao G. L., L. D. Liu, X. S. Zou, W. X. Chen (2007) Expression of KiSS-1, matrix metalloproteinase-9, nuclear factor-kappaBp65 in ovarian tumour, Zhonghua Fu Chan Ke Za Zhi, **42**, 34–38 (in Chinese).

[9] Zhu C., C. Takasu, Y. Morine, Y. Bando, T. Ikemoto et al. (2015) KISS1 associates with better outcome via inhibiting matrix metalloproteinase-9 in colorectal liver metastasis, Ann. Surg. Oncol., **22**, 1516–1523.

[10] Shengbing Z., L. J. Feng, W. Bin, G. Lingyun, H. Aimin (2009) Expression of KiSS-1 gene and its role in invasion and metastasis of human hepatocellular carcinoma, Anat. Rec. (Hoboken), **292**(8), 1128–1134.

[11] Zhang Y., Y. J. Tang, Z. H. Li, F. Pan, K. Huang et al. (2013) KiSS1 inhibits growth and invasion of osteosarcoma cells through inhibition of the MAPK pathway, Eur. J. Histochem., **57**, e30.

[12] Jarzabek K., M. Koda, L. Kozlowski, R. Milewski, S. Wolczynski (2015) Immunohistochemical study of KiSS1 and KiSS1R expression in human primary breast cancer: Association with breast cancer receptor status, proliferation markers and clinicopathological features, Histol. Histopathol., **30**(6), 715–23.

[13] Jarząbek K., L. Koziowski, R. Milewski, S. Woiczycski (2012) KiSS1/GPR54 and estrogen-related gene expression profiles in primary breast cancer, Oncol. Lett., **3**(4), 930–934.

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