Abstract. Background: KiSS-1 is a metastasis suppressor gene encoding a neuropeptide with potent antimetastatic activities in tumour cell lines. The transcriptional activity of the gene and its associations in resected breast cancer were analysed. Materials and Methods: Tumour messenger RNA (mRNA) of the KiSS1 exon I/II boundary was extracted from paraffin-embedded stage II or III node-positive breast adenocarcinomas of 272 women. KiSS1 mRNA was examined for associations with outcome, disease and molecular characteristics. Results: Only 8 out of 272 tumours (3%) yielded detectable KiSS1 mRNA levels. There was no evidence of correlation of KiSS1 transcription with the number of involved axillary nodes, grade, hormone receptor status or tumour size. Of women with increased KiSS1 mRNA tumour levels, 87.5% were postmenopausal, whereas only 48% were postmenopausal among patients without detectable KiSS1 mRNA (p=0.03). No association of KiSS1 transcription was found with transcription of the cell cycle-regulators HER2, VEGF, p53, BCL2, PAEP, or BIRC5. At a median follow-up of 62 months, there was no statistically significant difference between women harbouring KiSS1 mRNA-negative versus-positive tumours in terms of disease-free and overall survival (log-rank test p=0.54 and p=0.55, respectively). Conclusion: The metastasis suppressor gene KiSS1 is silenced in the vast majority of resected node-positive breast adenocarcinomas. These findings support the antimetastatic role of the gene and warrant its study as a prognostic marker and a therapeutic target.

Breast cancer is the most common malignancy and the leading cause of cancer-related mortality among women in most developed countries. Despite progress achieved with adjuvant combination chemotherapy, approximately half of all women with resected early breast carcinoma eventually relapse (1). The vast majority of breast cancer-related deaths result from systemic dissemination of tumour cells rather than primary tumour growth. The metastatic spread of malignant cells is a multi-step process that requires detachment from the primary site, survival in the circulation, attachment to and invasion of distant tissues, proliferation and angiogenesis at the secondary sites (2). Over the last decade, basic research has identified a handful of metastasis suppressor genes (MSG) that block any one of these steps, thus inhibiting formation of metastasis without affecting tumorigenicity or primary tumour growth (3). One such gene, KiSS1, has been cloned in chromosome 1q32-41. The gene is made up of four exons (I-IV) and encodes a hydrophobic 145-amino acid protein with potent antimetastatic activity in breast, bladder, pancreatic and esophageal cancer cell lines. Loss of KiSS1 expression correlated with systemic spread in solid tumours and with adverse patient outcome, making it a potentially useful prognostic factor, as well as a molecular target for therapeutic interventions (4, 5). We sought to analyse the gene’s transcriptional activity in 272 women with resected node-positive breast cancer and examine its associations with patient and tumour clinical and molecular characteristics, as well as relapse and survival.

Materials and Methods

Tumour tissue messenger ribonucleic acid (mRNA) from formalin-fixed paraffin-embedded (FFPE) biopsy specimens was collected from 272 patients with resected early (stage II-III) mostly node-positive breast adenocarcinomas. These patients had received dose-dense adjuvant chemotherapy of epirubicin, CMF (cyclophosphamide, methotrexate, 5-fluouracil) and paclitaxel at Hellenic Cooperative Oncology Group (HcCOG) centres from June 1997 until November 2000 (6). All patients had undergone modified radical mastectomy or breast-conserving surgery plus level I/II axillary node dissection. Patient characteristics are shown in Table I.

Five sections 10-μm thick were cut from each paraffin block. For all tumour samples included in the analysis the number of malignant cells represented at least 75% of all nucleated cells as judged by haematoxylin-eosin staining. Messenger RNA from mixed human reference total RNA pooled from ten human cell lines...
KiSS1 mRNA levels were detected in only eight among 272 patients (3%). Two hundred and sixty-four women harboured breast adenocarcinomas with no detectable KiSS1 transcription on RT-PCR. There was no evidence of any relationship of KiSS1 transcription with the number of involved axillary lymph nodes, histological grade, hormone receptor status or tumour size. Though the number of cases with KiSS1 transcription was too low to allow reliable subgroup analyses, seven out of eight patients (87.5%) with increased KiSS1 mRNA tumour levels were postmenopausal, whereas only 48% were postmenopausal among patients without detectable KiSS1 mRNA ($p=0.03$). KiSS1 mRNA-positive tumours were hormone receptor-negative and of poor differentiation more often than those tumours not expressing KiSS1 mRNA, though the finding did not have any statistical significance. In view of the restricted number of KiSS1 mRNA-positive tumours, this observation may be due to chance and should be interpreted with caution. KiSS1 mRNA status according to clinicopathological parameters is shown in Table II.

No association of KiSS1 transcription was found with transcription of other molecular variables tested (Table III). In particular, we determined mRNA levels of HER2 (human epidermal growth factor receptor type 2), VEGF (vascular endothelial growth factor), p53, BCL2, PAEP (glycodelin) and BIRC5 (survivin). Gene transcription was categorised as negative or positive according to mRNA expression when the 40-CT KiSS1 score was higher than zero.

Results

KiSS1 mRNA expression was categorised as negative or positive according to mRNA expression when the 40-CT KiSS1 score was higher than zero.
metastasis-suppressor gene. 

**KISS1** mRNA-positive tumours seemed to contain pro-proliferative HER2 mRNA more commonly and anti-apoptotic BCL2 mRNA less often than tumours negative for **KISS1** mRNA. However, these findings did not reach statistical significance and in view of the small sample size of **KISS1** positive cases, they can only serve as hypothesis-generating hints, at best.

After a median follow up of 62 months (range 1-86 months), forty-four out of 272 patients had died (16%) (Table IV). The proportion of deaths was 16% among those patients harbouring tumours with no expression of **KISS1** mRNA versus 25% among those bearing tumours with **KISS1** mRNA expression. Seventy-six patients out of the total of 272 (28%) had demonstrated malignant relapse locoregionally or distantly. Among patients affected by **KISS1** mRNA-negative tumours, 28% relapsed versus 37.5% among those with **KISS1** mRNA-expressing tumours. The difference in deaths and/or malignant relapse between patients with **KISS1**-positive and -negative mammary cancer was not statistically significant. Overall survival and disease-
free survival did not differ significantly between the two
groups (log-rank p=0.55 and p=0.54, respectively), with
KiSS1 transcriptional status failing to show prognostic
significance (Figure 1).

Discussion

Breast cancer metastasis seems to be regulated by the
interplay of metastasis-promoter (Ras, MEK1, PKC,
osteopontin, chemoattractants, proteinases, adhesion
molecules) and metastasis-suppressor genes (E-cadherin,
Nm23, TIMPs, KAI1, KiSS1, Maspin, Mkk4, BRMS1) (7).
Among the fourteen known metastasis-suppressor genes
(MSG), KiSS1 is the only one that binds a G-protein coupled
receptor (GPR54 or AXOR12 or hOT7T175) and is believed
to act late in the metastatic cascade by preventing growth of
the metastatic deposit, as opposed to early metastasis-
suppressor genes (Nm23, KAI1) that suppress cell
detachment and migration from the primary tumour (7,8).

The KiSS1 gene consists of four exons, the first two not
translated. Exon III contains the translational start site
followed by 103 translated bases, while exon IV is the
largest, consisting of 335 translated and 121 non-translated
bases (4). The encoded full-length 145-amino acid KiSS1
protein undergoes post-translational cleavage at dibasic sites
such as R<sup>66</sup>-R and K<sup>123</sup>-R resulting in the active 54-amino
acid peptide metastin or kisspeptin-54 (KP54) (9-11).
Multiple shorter products, collectively called kisspeptins,
result from naturally occurring proteolytic cleavage. The
kisspeptins that retain the last 10 carboxy-terminal amino
acids are able to bind the receptor GPR54 for effecting
KiSS1 actions (12).

The KiSS1 protein is normally expressed in the placenta,
testes, brain and spinal cord, suggesting a role for regulation
of trophoblastic invasion, and of pubertal and
neuroendocrine development (8, 9, 13). In vitro data
identifying KiSS1 as a putative MSG were confirmed when
suppression of cellular invasion and metastasis was seen in
melanoma, breast and bladder cancer cell lines as well as in
nude mice upon neoplastic clone transfection by KiSS1
cDNA (14-17). Subsequently, low KiSS1 mRNA expression
was found to correlate with venous invasion, advanced clinical
stage, occurrence of metastases and recurrence in
retrospective patient series with melanoma, gastric, bladder,
estophageal, pancreatic and endometrial cancer (18-21).
Recently, brain metastases from breast cancer were shown to
have low KiSS1 mRNA and protein levels in comparison to
the breast primary and the normal mammary tissue (22).
The molecular pathways through which KiSS1 exerts its
antimetastatic effects have not yet been elucidated (8-10, 23-
25). E-cadherin up regulation and reduction of matrix
metalloprotease 9 (MMP9) expression, increased intracellular
calcium release and inhibition of protein kinase C, PI3K-
AKT pathway blockade resulting in induction of apoptosis,
modulation of the MAPK pathway and reduction of NF-κB
p50/p65 heterodimer formation have been reported as KiSS1
effects in neoplastic cell cultures.

In our study, we report for the first time absence of
detectable KiSS1 transcription in a cohort of 272 women
with node-positive, resected breast adenocarcinoma.
Silencing of the KiSS1 MSG is compatible with the
epidemiology of our patient population: more than three-
quarters of our patients had a high-volume (≥4 nodes)
axillary nodal tumour burden, a surrogate marker of the
tumour propensity for metastatic dissemination. The
silencing of an MSG could allow malignant cells to switch to
a ‘metastasis-capable’ phenotype characterized by migration,
survival in the lymphatic/venous circulation, invasion-homing
in regional lymph nodes and growth at secondary sites. Our
observation is in keeping with published findings of reduced
KiSS1 mRNA levels in advanced-stage solid tumours (18-22).
Of note, in our cohort, KiSS1 transcription was not related to
transcription of other genes such as survivin, glycodelin,
HER2, p53, or BCL2, suggesting that the metastasis-
suppressive action of KiSS1 may be effected independently
of those pivotal cell cycle-regulatory genes. The increased
percentage of postmenopausal women in the
KiSS1-positive group may offer a biological explanation of the
more indolent behaviour of breast cancer in this group in
comparison to younger women. Unfortunately, the small
group size of KiSS1-expressing tumours did not allow for
reliable analysis of its prognostic significance. Possible
molecular mechanisms of KiSS1 down-regulation include homozygous deletion, promoter methylation and transcription factor deletion or inactivation (20). Recently Mitchell et al. established the induction of KiSS1 transcription by binding of the activator protein-2 alpha (AP2a)/specificity protein-1 (Sp1) complex to the gene’s promoter (26). The AP2a transcription factor is encoded by a gene in chromosome 6p and interestingly, chromosome 6 loss of heterozygosity has been associated with loss of KiSS1 expression and dissemination of gastric cancers and melanomas, while introduction of an intact chromosome 6 in melanoma cell lines suppressed metastases (27). Recently, we identified a KiSS1 exon IV point mutation substituting guanine for cytosine, 242 bases from the translation start site, resulting in substitution of the hydrophobic amino acid arginine (P81R) and consequently in modification of the tertiary stereotactic structure of the KiSS1 protein. The mutation was harboured by 17 out of 50 women with early breast cancer and correlated with high-volume axillary nodal metastases (28). In sharp contrast to our findings, Martin et al. reported increased KiSS1 mRNA and protein levels in breast carcinomas of 124 patients, especially in those with high grade, node positive tumours and concluded that KiSS1 is associated with poor prognosis and metastatic dissemination (29). Still, the primers used for RT-PCR mRNA analysis by the investigators may have flanked different sequences, making interpretation of discrepant results difficult. Moreover, immunohistochemistry was performed on cryostat sections that may contain more than 50% healthy tissues, while the antibody used is not specified. Finally, the possibility of mRNA detection of mutant inactive KiSS1 should also be contemplated. Indeed, as the authors state in their manuscript, reduced levels of KiSS1 receptor were associated with adverse outcome in the 124 patients with breast cancer.

We present for the first time data showing functional silencing of KiSS1 in patients with high-risk early breast cancer. Our results confirm previously published preclinical and clinical evidence supporting the metastasis-suppressor role of the gene. Study of KiSS1 transcriptional activity in patients with node-negative breast cancer, breast cancer primaries and metastatic deposits as well as in patients with other solid tumours is mandatory. As this molecular alteration seems to occur relatively early in tumours at localised stages, clinical research towards development of MSG agonists or restoration of KiSS1 function holds promise for arresting micrometastatic growth and preventing malignant relapse in cancer patients.

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