The Missing Kiss of Life: Transcriptional Activity of the Metastasis Suppressor Gene KiSS1 in Early Breast Cancer

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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-fluorouracil) and paclitaxel at Hellenic Cooperative Oncology Group (HeCOG) 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 hematoxylin-eosin staining. Messenger RNA from mixed human reference total RNA pooled from ten human cell lines

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Table I. *Basic patient characteristics.*

N=272	N (% percentage)
Age (median)	50 years
Range	22.5-76 years
Menopausal status	
Premenopausal	139 (51%)
Postmenopausal	133 (49%)
Stage (Pathologic)	
II	65 (24%)
III	207 (76%)
Tumour size	
T1	83 (30.5%)
T2	136 (50%)
T3	52 (19.1%)
Unknown	1
Nodal status	
Negative	1
1-3 nodes	63 (23.2%)
4 or more nodes	208 (76.5%)
Grade	
1-2	133 (49%)
3-4	138 (51%)
Unknown	1
Hormone receptor status	
Negative	58 (21.3%)
Positive	210 (77.2%)
Unknown	4

(Stratagene, La Jolla, CA, USA) was used as positive control. RNA-free DNA extracted from tumour tissues was used as negative control. Transcriptional expression of housekeeping genes (reference genes GAPDH and RPL37A) were used as controls. KiSS1 mRNA from FEPE tumors was extracted by means of an experimental method based on proprietary magnetic beads from Bayer HealthCare Diagnostics (Leverkusen, Germany) In short, the FFPE slide was deparraffinized in xylol and ethanol, the pellet was washed with ethanol and dried at 55˚C for 10 min. The pellet was then lysed and proteinized overnight at 55˚C with shaking. After adding a binding buffer and magnetic particles (Bayer HealthCare Diagnostics Research, Leverkusen, Germany) nucleic acids are bound to the particles within 15 min. at room temperature. On a magnetic stand the supernatant was removed and beads washed several times with washing buffer. After adding elution buffer and incubating for 10 min at 70˚C the supernatant was removed on a magnetic stand without touching the beads. After standard DNAse I treatment for 30 min at 37˚C and inactivation of DNAse I, the solution is used for RT-PCR.

The primers used for RT-PCR were located in untranslated exons I and II (KiSS1 S6R 5'UTR exon I forward primer AGGTGGTCTCGTCACCTCAGA and KiSS1 S6R 5'UTR exon II reverse primer TGAGAAGAGGCAGGTCCTAGAAGT), while the RNA-specific probe over the exon I/II boundary used was KiSS1 S6R 5'UTR CCAGGCCAGGACTGAGGCAAGCCTCAA. The quality and quantity of RNA was checked by measuring absorbance at 260 nm and 280 nm. Pure RNA has an A260/A280 ratio of 1.9-2.0. Transcriptional activity of the genes was assessed with quantitative Reverse Transcriptase Taqman™ polymerase chain reaction (RT-PCR) analysis. Forty cycles of nucleic acid amplification were applied and GAPDH and RPL37A used as housekeeping genes at a cycle threshold (CT) of 28. A normalised 40-CT KiSS1 score was calculated that correlates proportionally to RNA transcription levels. Results were categorised as no *KiSS1* mRNA expression when the normalized KiSS1 score was negative or zero and as presence of *KiSS1* mRNA expression when the KiSS1 score was higher than zero.

Statistical analysis. Associations of KiSS1 transcriptional activity with clinical and molecular characteristics of the patient and tumour were examined by the chi-square and Fisher's exact tests. Overall survival (OS) and disease-free survival (DFS) were calculated from time of diagnosis to death or last follow-up and to malignant relapse, death without relapse or last follow-up, respectively. Survival curves and comparison by KiSS1 transcriptional status were calculated with the Kaplan-Meier product-limit method and the Log rank test. All p-values are two-sided and observed differences are considered statistically significant when $p<0.05$.

Results

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 in comparison to reference gene expression. These regulators are key molecules for the control of cellular proliferation, angiogenesis, DNA repair and apoptosis. Still, their transcription was not found to be associated with the transcriptional status of the *KiSS1*

Table II. *Patient and tumour characteristics by KiSS1 transcriptional status.*

	KISS1 mRNA expression			
	No (N=264)		Yes (N=8)	
Age (years)				
Median	51		59	
Range	22.5-76		39.5-62	
	N	%	N	%
Menopausal status 1				
Premenopausal	138	52	1	12.5
Postmenopausal	126	48	7	87.5
Treatment group				
E-T-CMF	119	45	4	50
E-CMF	145	55	4	50
Type of surgery				
MRM	211	80	7	87.5
PM	53	20	1	12.5
Receptor status				
Negative	54	20	4	50
Positive	206	78	4	50
Unknown	4	1	-	-
Positive nodes				
1-3	61	23	2	25
≥4	202	76	6	75
Negative	1	0.4	-	-
Grade				
I-II	130	49	3	37.5
III-IV	133	50	5	62.5
Unknown	1	0.4	-	-
Tumor size				
≤2 cm	81	31	2	25
2-5	131	50	5	62.5
>5	51	19	1	12.5
Unknown	1	0.4	-	-

The proportion of post-menopausal women was significantly higher in patients with expression of *KISS1* mRNA ($p=0.03$).

Table III. *Transcription of key molecular variables by KiSS1 mRNA status.*

	KISS1 mRNA expression			
	No (N=264)		Yes (N=8)	
	N	%	N	%
PAEP				
Not-expressed	198	75	6	75
Expressed	66	25	2	25
BIRC5				
Not-expressed	9	3	-	-
Expressed	255	97	8	100
	KISS1 expression			
	No		Yes	
HER2 (N=236)	(N=229)		(N=7)	
Not-expressed	167	73	4	57
Expressed	62	27	3	43
VEGF (N=220)	(N=212)		(N=8)	
Not-expressed	57	27	2	25
Expressed	155	73	6	75
p53 (N=242)	(N=234)		(N=8)	
Not-expressed	180	77	5	62.5
Expressed	54	23	3	37.5
Bcl-2 (N=244)	(N=236)		(N=8)	
Not-expressed	120	51	6	75
Expressed	116	49	2	25

No association of KISS1 expression with molecular variables was found ($p>0.05$ in all cases).

Table IV. *Survival data by KiSS1 transciptional status.*

	KISS1 mRNA expression	
	No (N=264)	Yes (N=8)
Survival (months)		
Events	42	2
Range	1 – 86+	35 – 78+
Median	Not reached	78
Disease-free survival (months)		
Events	73	3
Range	1 – 83+	15 – 71+
Median	Not reached	Not reached

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^{66}-R and K^{123}-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, esophageal, 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-kB p50/p65 heterodimer formation have been reported as *KiSS1* effects in neoplastic cell cultures.

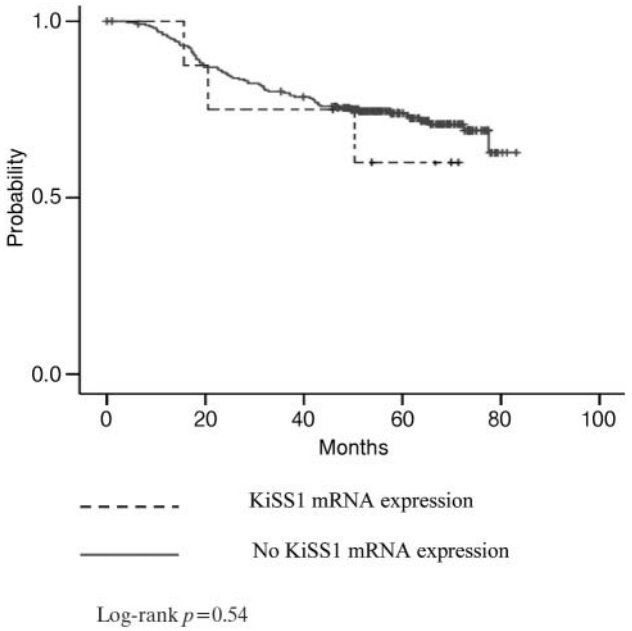

Figure 1. *Disease-free survival of 272 early breast cancer patients by KiSS1 transcriptional status.*

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 proline by the polar, hydrophilic 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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