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A twisted kiss: in vitro and in vivo evidence of genetic variation and suppressed transcription of the metastasis-suppressor gene KiSS1 in early breast cancer.

G. PENTHEROUDAKIS[1], L. KOSTADIMA[1], L. DOVA[2], I. GEORGIOU[3], T. TZAVARAS[4], G. VARTHOLOMATOS[2], R. M.WIRTZ[5], G. FOUNTZILAS[6], V. MALAMOU-MITSI[7], N. PAVLIDIS[1]

[1]Department of Medical Oncology, Ioannina University Hospital, Greece, e-mail:npavlid@uoi.gr. [2]Molecular Biology Unit, Hematological Laboratory, Ioannina University Hospital, Greece, [3]Genetics Unit, Department of Obstetrics and Gynecology, Ioannina University Hospital, Greece, [4]Department of General Biology, Medical School, University of Ioannina, Greece, [5]Siemens Medical Solutions Diagnostics GmbH, Cologne, Germany, [6]Department of Medical Oncology, Papageorgiou General Hospital, Aristotle University of Thessaloniki School of Medicine, Greece. [7]Department of Pathology, Ioannina University Hospital, Greece.

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KiSS-1 is a metastasis suppressor gene, its inactivation linked to advanced tumor stage and dismal prognosis. We studied its mutational status , transcription and protein expression in human cancer cell lines and patients with early breast cancer.

Tumor tissue DNA and messenger RNA (mRNA) of KiSS1 exons III and IV from the human cancer cell lines Hela, Jurkat, A549, W138t, MCF-7 and from formalin-fixed resected breast adenocarcinomas from 50 women were analysed by means of PCR-SSCP, RT-PCR and sequencing. Tumor tissue was stained for KiSS1 protein expression by means of the streptavidin-biotin complex immunoperoxidase assay. Presence of KiSS1 mutation, mRNA levels and protein staining were examined for correlations with patient/tumor characteristics.

A transversion in exon IVa replacing cytosine with guanine was identified 242 base pairs from the translation start site (242C>G) in the cell lines MCF-7, A549 and in 5/50 tumors (10%), resulting in substitution of proline by arginine (P81R) and alteration of the protein tertiary structure. As the substitution was present in germ-line DNA in 3/5 breast cancer patients harbouring the polymorphism in their tumor, the incidence of tumour-specific somatic mutation was 4% among the 50 patiens with early breast cancer. Although the P81R substitution was associated with reduced KiSS1 protein immunoreactivity (56% in wild-type tumors versus 20% in KiSS1-variant tumours) and with axillary nodal involvement (55% in wild-type versus 80% in KiSS1-variant tumors), the correlations did not reach statistical significance. KiSS1 mRNA was detected in only 15/48 tumours (31%) and showed no correlation with mutation or protein expression. Twenty-six tumors stained for KiSS1 protein, in contrast to the universal strong staining seen in normal breast parenchyma and placental tissues. At a median follow-up of 38 months, relapses occurred in 20% of women with non wild-type tumors versus 13% of women with wild-type KiSS1 tumors (p=0.7). Presence of KiSS1 mutation, mRNA levels and protein expression did not have prognostic significance for relapse-free survival.

In conclusion, altered nucleotide sequence and repression of transcription are two potential mechanisms of suppression of the anti-metastatic effects of KiSS1 in early breast cancer: Confirmation in larger cohorts and study of functional effects of the 242C>G exon IVa mutation are warranted.

Key words: KiSS1, metastasis-suppressor gene, breast cancer, mutation, transcription.

Breast cancer is the leading cause of cancer-related mortality among women in most developed countries. Despite progress achieved with adjuvant chemotherapy, approximately half of all women with resected early breast carcinoma eventually experience systemic malignant relapse that is ultimately fatal [1]. The metastatic spread of malignant cells is a multi-step process that requires detachment from the primary site and systemic dissemination, attachment to distant tissues, invasion, proliferation and angiogenesis at the secondary sites [2]. Over the last decade, basic research has identified metastasis suppressor genes that block each of these steps, thus inhibiting formation of metastasis without affecting primary tumor growth [3]. One such gene, KiSS1, has been cloned in chromosome 1q32-41. The gene is made up from four exons (I-IV) and encodes a hydrophobic 145-aminoacid protein with potent antimetastatic activity in breast, bladder, pancreatic and esophageal cancer cell lines [4].

In retrospective patient series with various solid tumours, the loss of KiSS1 expression at the mRNA and protein level was associated with systemic spread and adverse outcome [5]. We sought to screen for mutation, study the transcription of the translated gene exons III-IV and tissue expression of the relevant protein in five commonly used human cancer cell lines, followed by a cohort of 50 patients with early breast cancer. Moreover, we examined possible correlations of KiSS1 molecular data with patient and tumor characteristics.

Materials and methods

Tumor tissue from formalin-fixed paraffin embedded (FFPE) biopsy specimens was collected from 50 patients with resected early (stage I-IIIA) breast adenocarcinomas diagnosed at the Department of Medical Oncology, Ioannina University Hospital from January 2002 until November 2004. Patient characteristics are shown in Table 1. Patient and tumor characteristics were retrieved from the patients` case sheets for study of correlations with molecular data. The study was approved by the local institutional ethics committee.

Human cancer cell lines. In order to examine the KISS1 genetic integrity in different human cell lines we screened and analysed by means of PCR/sequencing the exon III-IV entire translating sequence that is coded by 438 bp. Exon IV, consisting of 335 translated and 121 non-translated bases, was split for analysis in two fragments (IVa and IVb), in order to achieve high screening and sequencing accurary. The following primers, (Invitrogen-Life Technologies Merelbeke, Belgium), were used for PCR amplification.

Exon3: Forward 5'-CTC AGC CTC AAG GCA CTT CT-3'
Reverse 5'-CAC TCC TTT CCC CAG AGG AT-3'.
Exon4a: Forward 5'-TCC TAG GCC AGC AGC TAG AA-3'
Reverse 5'-CCA GTT GTA GTT CGG CAG GT-3'.
Exon 4b: Forward 5'-ACC TGC CGA ACT ACA ACT GG-3'
Reverse 5'-TCT TTT ATT GCC TCG GGT TG-3'.

Table 1. Basic patient characteristics

	N=50 (% percentage)
Age (median)	55 years
Stage (Pathologic)	
I	4 (8%)
II	18 (36%)
III	28 (56%)
Nodal status	
Negative	18 (36%)
1-3 nodes	11 (22%)
4 or more nodes	21 (42%)
Grade	
1-2	33 (67%)
3-4	17 (33%)
Hormone receptor status	
Negative	15 (31%)
Positive	35 (69%)

All human cell lines were obtained from the American Type Culture

Collection (ATCC, Manassas, VA) and were cultured as recommended. We screened five human cancer cell lines: Hela, Jurkat , A549, W138t and MCF-7.

Genomic DNA isolation from cell lines was performed using the QIAGEN DNEasy TM tissue kit (Westburg, Leusden, The Netherlands) according to manufacturer`s instructions. PCR was performed in a total volume of 50μl, containing 50ng of genomic DNA, 10X PCR buffer, 1.5 to 2.5mM $MgCl_2$, each dNTP at 0.2mM, each primer at 0.2μM and 1 Unit of Taq DNA Pol (Taq Core Kit 10, Q-BIOgene). Thermal cycler conditions were consisted of an initial denaturation step at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 5 min, annealing at 55° to 61°C for 1 min, extension at 72°C for 30 sec and a final extension step at 72°C for 7 minutes, in a gradient thermocycler (PTC 200 Peltier thermal Cycler, MJ Research USA). A total of 10 μl of the PCR products was mixed with an equivalent volume of denaturing loading buffer (98% formamide, 0.005% xylene cyanol and bromophenol blue). The samples were denatured at 95°C for 10 min, put on ice for 5 min and run at 4°C in a 10% native polyacrylamide gel containing 10% (v/v) glycerol, at 12W for 16-20 hours. PCR products showing an aberrant single stranded conformation pattern were re-amplified and sequenced in both directions using the BigDye Terminator v.3.1 Cycle Sequencing Kit on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Forester City, CA, USA).

Immunohistochemical assay. KiSS1 protein was evaluated immuno- histochemically by means of the streptavidin-biotin complex immunoperoxidase assay on 5 μm sections from formalin-fixed paraffin-embedded tissue in the previously described 50 cases of breast cancer. Ten placental samples and normal breast parenchyma were also stained as positive controls. The slides were immunostained with a polyclonal rabbit anti-KiSS1 IgG antibody (FL-145, Santa Cruz Biotechnologies, US) diluted at 1:300, using streptavidin-biotin complex autostainer (Labvision Corp, Fremont, CA) with diaminobenzidine as chromogen. The antibody is raised against a recombinant protein corresponding to aminoacids 1-145 representing full-length KiSS1 of human origin.

The immunohistochemical (IHC) KiSS1 expression was evaluated semi-quantitatively based on the intensity of staining (0,+1, +2, +3) and the percentage of positive tumor cells (0-100%). The total score was calculated by multiplying the intensity grading by the relative percentage of tumor cells, ranging from 0 to 300. The IHC score was subsequently categorized as negative (0-10), weakly positive (11-50), moderately positive (51-150) or strongly positive (151-300).

Molecular analysis for KiSS1 gene mutations in cohort of 50 breast tumors.

10-μm tissue sections of specimens with malignant cellularity of at least 75% were scraped from the slide for DNA isolation and mutational analyses of exons III, IVa and IVb. Genomic

DNA was extracted from fifty formalin-fixed, paraffin-emdedded breast cancer tumor specimens, from peripheral blood lymphocytes of breast cancer patients positive for any KiSS1 nucleotide substitution in their tumor and from peripheral blood lymphocytes of fifty healthy control subjects according to standard procedures. Genetic analysis of the KiSS1 gene was performed by polymerase chain reaction (PCR) amplification of exons III and IV, mutational screening by means of Single Stranded Conformational Polymorphism (SSCP) followed by sequencing of PCR products in all 50 breast tumors. The primers and procedures used for SSCP and sequencing were as previously mentioned in the cell line experiments. Sequencing of KiSS1 exons III/IV was also performed by Macrogen Inc, Seoul, Korea, for independent external validation of sequencing results from our local laboratory.

Molecular analysis for KiSS1 gene transcription. KiSS1 mRNA from formalin fixed paraffin embedded tumors was extracted by means of an experimental method based on proprietary magnetic beads from Siemens HealthCare Diagnostics GmbH. In short, the FFPE slide is deparraffinised in xylol and ethanol, dried, lysed and proteinized overnight at 55°C. After adding a binding buffer and the magnetic particles (Siemens HealthCare Diagnostics, Cologne, Germany) nucleic acids are bound to the particles, the supernatant taken away on a magnetic stand and the beads washed several times. After normal DNAse I treatment for 30 min at 37°C and inactivation of DNAse I the solution is used for RT-PCR. The RNA-specific primers used were located across the exon/exon boundaries of exon III and IV (KiSS1 P2 5`UTR exon 3 forward primer TCTGTGCCACCCACTTTGG and KiSS1 P2 5`UTR exon 4 reverse primer CAGGAGGCCCAGGGATTCT), while the RNA-specific probe span the exon III / IV boundary (KiSS1 S6R 5`UTR TCTAGACCCACAGGCCAGCAGCTA). The quality and quantity of RNA was checked by measuring absorbance at 260 nm and 280 nm. Transcriptional activity of the genes was assessed with quantitative Reverse Transcriptase Taqman™ polymerase chain reaction (RT-PCR) analysis. We applied 40 cycles of nucleic acid amplification and used GAPDH and RPL37A as housekeeping genes, while adjusting the amount of RNA put into each reaction at a cycle threshold (CT) of 23. We calculated a normalized 40-CT KiSS1 score that correlates proportionally to RNA transcription levels. Results were categorized 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. The correlation of KiSS1 molecular data with characteristics of the patient and tumour was examined by the chi-square and Spearman`s Rho test. Relapse-free survival (RFS) was calculated from time of diagnosis to relapse, death or last follow up. Survival curves were calculated with the Kaplan-Meier product-limit method and the Log-rank test. All p-values are double-sided and observed differences are considered statistically significant when $p<0.05$.

Results

KiSS1 mutational screening in human cancer cell lines. We screened three types of epithelial like cells, human cervix epitheloid carcinoma cells (HeLa), human breast adenocarcinoma cells (MCF-7) and human lung carcinoma cells (A549). We also used lymphoblasts from Jurkat human acute T-cell leukemia line and W138 SV40-transformed human lung fibroblasts.

The SSCP analysis revealed that KISS1 exon IVa in MCF-7 and A549 cell lines had a different electrophoretic mobility compared to KISS1 exon IVa in HeLa, Jurkat and W138 transformed cells. The aberrant SSCP bands were subsequently sequenced and found to harbour a novel C to G substitution in KISS exon IVa which is a cytosine to guanine substitution 242 base pairs from the translation start site. This point mutation changes a CCC codon to CGC, resulting in substitution of the hydrophobic proline by the hydrophilic arginine (P81R) in the wild type amino-acid sequence.

KiSS1 mutational screening and correlations in 50 resected breast carcinomas. We were unable to observe exon III or IVb point mutations, insertions or deletions in any sample, as no shifted bands (aberrant patterns) were detected in any of the 50 breast cancer samples neither in the 50 healthy control peripheral blood samples. However, when exon IVa was screened on SSCP polyacrylamide gel electrophoresis, a shifted band was recorded in 5 of 50 (10%) breast cancer samples as opposed to none of the 50 healthy controls. However, the shift was present in the peripheral blood sample in 3 out of 5 breast cancer patients who harboured tumors with the exon IVa shift, indicating presence of a germ-line polymorphism rather than tumor-specific somatic mutation in those three patients. Accordingly, the incidence of tumor-specific KiSS1 somatic mutation in our early breast cancer patient cohort was only 4% (2/50).

Sequence analysis in both local and external laboratories confirmed the presence of wild-type exon III, IVb sequences in all 50 breast tumors and wild-type exon IVa sequence in 45. The same five breast tumors harboured the cytosine to guanine substitution 242 base pairs from the translation start site (242 C>G), previously observed in cancer cell lines MCF7 and A549. This polymorphism was indeed present in DNA from peripheral blood lymphocytes in three of five patients and was restricted to the tumor in only two of five patients. This single nucleotide substitution , previously reported as RS4889 391C>G in the NCBI Databank, changes a CCC codon to CGC, resulting in the replacement of the aminoacid proline by arginine (P81R) in the predicted wild-type aminoacid sequence previously reported as HSU43527: MNSLVSWQLLLFLCATHFGEPLEKVASVGNSRPTGQQLESLGLLAPGEQSLPCTERKPAATARLSRRGTSLSPPPESSGS**P**QQPGLSAPH
SRQIPAPQGAVLVQREKDLPNYNWNSFGLRFGKREA.
APGNHG RSAGRGWGAGAGQ.

A characteristic example of exon IVa SSCP aberrant bands in amplified tumor DNA from the five samples on polyacry-

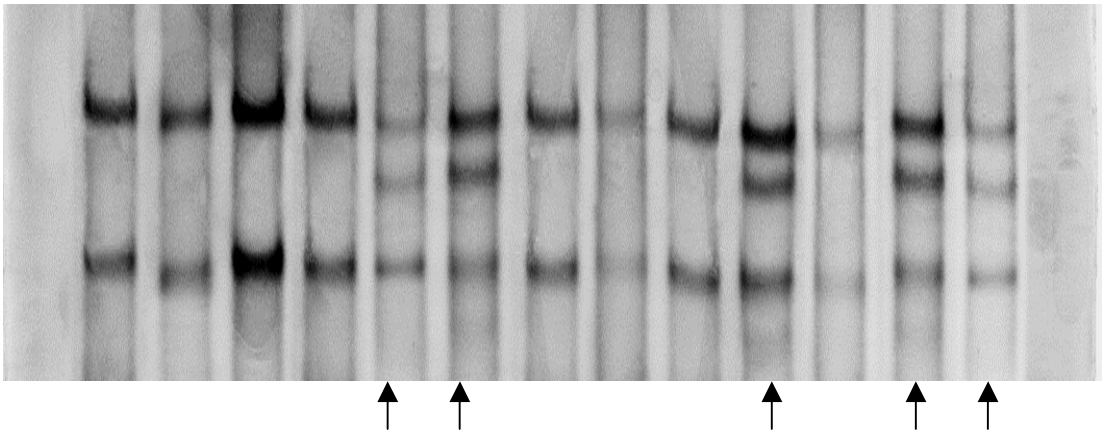
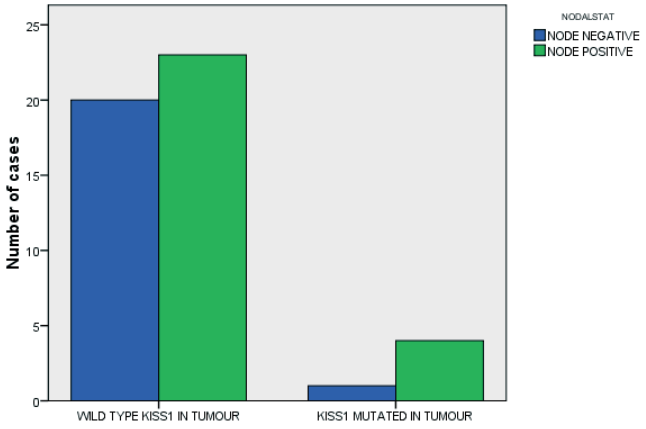


Figure 1. KiSS1 exon IVa SSCP aberrant bands in five samples on polyacrylamide gel electrophoresis

Table 2. Axillary nodal involvement by KiSS1 mutational status



lamide gel electrophoresis, the corresponding sequencing data and those of a wild-type exon IVa from a patient`s peripheral blood lymphocytes are given in Figures 1 and 2a, 2b respectively.

Although the presence of exon IVa KiSS1 nucleotide substitution did not correlate with protein expression from a statistical test perspective, an inverse association cannot be excluded: The proportion of positive immunohistochemical KiSS1 staining was 56% in wild-type tumors versus 20% in tumors harbouring variant KiSS1 (Spearmann correlation r=-0.21, p=0.14). Moreover, 80% of variant KiSS1 tumors involved axillary lymph nodes versus only 55% of wild-type KiSS1 tumours (Spearman correlation r=0.16, p=0.027) (Table 2). These correlations did not prove to be statistically significant either, though the small sample size precludes robust conclusions. A correlation was suggested even when high-volume nodal infiltration was considered: Among 45 patients with wild-type KISS1, only 33% had 4 or more nodes involved. In contrast, among 5 non-wild type KISS1 patients, 60% had 4 or more nodes involved. No correlation of KISS1 mutational status with HER 3+ overexpression (p=0.69), hormone-receptor status (p=0.15), menopausal status (p=0.69), age (p=0.5), histologic type (p=0.27) or histological grade (p=0.83) was found.

KiSS1 transcription and correlations. We were able to evaluate KiSS1 mRNA levels in 48 tumors. Detectable KiSS1 mRNA was present in 15 out of 48 tumors (31%), the remaining 33 tumors harbouring non-detectable KiSS1 mRNA at the exon III/IV boundary. KiSS1 transcription seemed to be similar in tumors bearing wild-type and variant KiSS1 (p=0.13). KiSS1 mRNA did not correlate with immunohistochemical protein expression either: mRNA positivity was seen in 9/25 (36%) tumors with KiSS1 protein expression and in 14/23 tumours with no KiSS1 protein staining (p=0.16). KiSS1 mRNA status did not correlate with HER2 overexpression, axillary nodal involvement, histological grade, patient age and menopausal status (p>0.05). Although the proportion of tumors involving ten or more axillary lymph nodes was 17% in KiSS1 mRNA (+) tumors versus 35% in KiSS1 mRNA (-) tumours, the difference was not statistically significant (p=0.25). There was a trend for inverse correlation of KiSS1 mRNA with tumour hormonal receptor status (56% of KiSS1 mRNA positive tumors were hormone receptor positive versus 84% of KiSS1 mRNA negative tumors, p=0.037). Finally, lobular adenocarcinomas made up 28% of KiSS1 mRNA (-) tumors versus only 4.5% of KiSS1 mRNA (+) tumours (p=0.033).

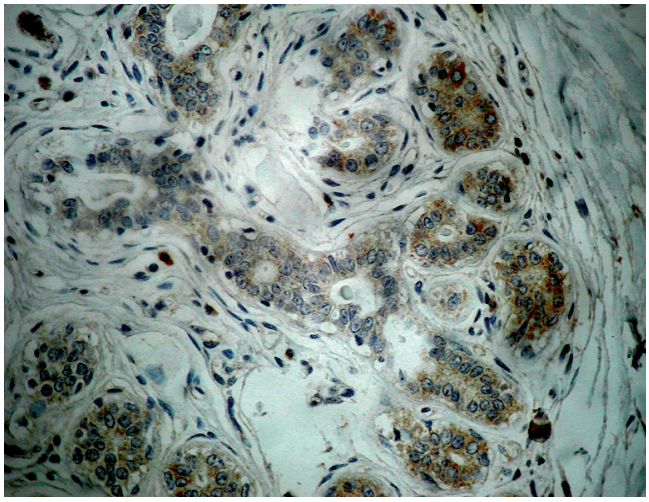
KiSS1 immunohistochemical expression and correlations. Twenty-four of 50 analyzed samples (48%) were graded as negative for KiSS1 expression, while staining was observed in 26 tumors (52% of examined cases). Twenty tumors were weakly positive (40%) and six moderately positive (12%) while no tumour showed strong KiSS1 staining. The immunostaining in positive samples was homogeneous cytoplasmic. In contrast to breast tumor samples, normal breast parenchyma and placental tissues exhibited strong cytoplasmic KiSS1 protein expression. Characteristic patterns of immunohistochemical KiSS1 protein expression in normal breast glandular tissue and in an invasive breast adenocarcinoma are

Figure 2. KiSS1 exon IVa mutated (a, position 101) and wild type (b) sequence.

shown in Figures 3a and 3b respectively. As stated earlier, KiSS1 protein staining showed a non-significant inverse correlation with mutated exon IVa KiSS1 but no association with KiSS1 mRNA levels. Immunohistochemical KiSS1 protein expression was not associated to HER2 overexpression, histological grade or type, axillary nodal involvement, hormone receptor, patient age or menopausal status.

Correlation of KiSS1 molecular data with outcome. At a median follow-up of 38 months, only six out of 50 women relapsed (12%). One relapse occurred among the five women harbouring tumors with variant KiSS1 (20%) while six in 45 women with wild-type KiSS1 tumors (13%). The number of events is too low, the sample size too small and the follow-up time too short to allow any reliable conclusions on survival analyses to be drawn

(a)



(b)

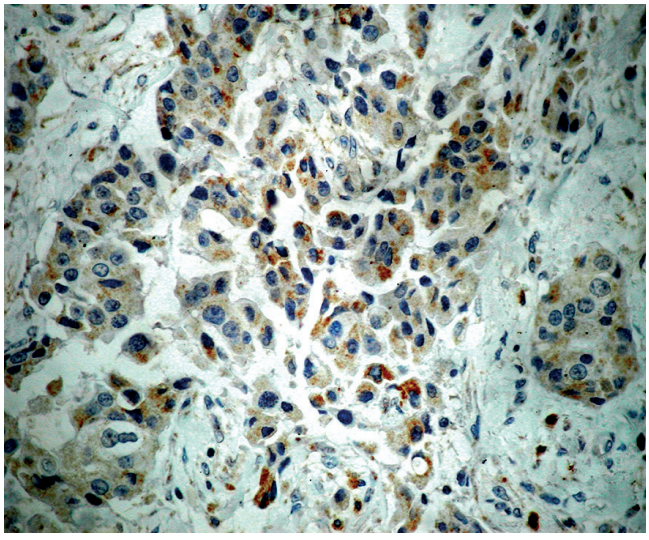


Figure 3. Imunohistochemical KiSS1 protein expression in (a) normal breast (strong) and (b) breast tumour (weak)

(Log-rank p=0.7). Disease relapse occurred in four out of 24 women with tumors not expressing KiSS1 protein (17%) versus two out of 26 women with KiSS1-staining tumors (8%, p=0.6). Neither KiSS1 mRNA status nor protein immunoreactivity exhibited any prognostic significance for relapse-free survival. Tumor grade, histological type, nodal status, patient age and menopausal status failed to show any prognostic significance in univariate regressional hazard analysis, though sample size and number of events limit the value of this observation.

Discussion

Breast cancer metastasis is regulated by the interplay of metastasis-promoter and metastasis-suppressor genes (E-cadherin, Nm23, TIMPs, KAI1, KiSS1, Mampsin, Mkk4, BRMS1) [6]. Among the 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 [6, 7]. The KiSS1 gene consists of four exons, the first two of which are 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-aminoacid KiSS1 protein contains a protein kinase C phosphorylation domain, a secretory signal, a polyproline rich region (SH3 ligand) and a number of motifs important for post-translational modifications. Post-translational cleavage at dibasic sites R^{66}-R and K^{123}-R produces the active 54-aminoacid 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 aminoacids are able to bind the receptor GPR54 for effecting KiSS1 actions [11].

The KiSS1 protein is normally expressed in the placenta, testis, brain and spinal cord, suggesting a role for regulation of trophoblastic invasion, of pubertal and neuroendocrine development [7, 8, 12]. Structural traits of the protein imply that it is a secreted neuropeptide that acts on target cells via the cell surface GPR54 G-protein coupled receptor in an autocrine and paracrine fashion [5]. Recently, KiSS1 has been identified as an unexpected molecular switch for puberty and various mutations of its receptor GPR54 (L148S, R331X, X399R, C223R, R297L, L102P) have been linked to the syndrome of idiopathic hypogonadotrophic hypogonadism. In vitro data identifying KiSS1 as a putative metastasis-suppressor gene (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 [13–16]. The human breast cancer MDA-MB-435 and the human melanoma C8161, MelJuSo cell lines were among the first ones reported not to express KiSS1 and respond to KiSS1 transfection with inhibition of the metastatic phenotype. We screened five common human cancer cell lines expressing KiSS1 and found that two, the human breast adenocarcinoma MCF7 and the human lung carcinoma A549 harbour an identical P81R (242C>G) substitution. This substitution results in replacement of a neutral, hydrophobic aminoacid (proline) by a polar, hydrophilic one (arginine), a change with profound structural effect as it modifies the tertiary stereotactic structure of the protein. If this alteration proves to be an inactivating one, the loss of function 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. In view of the available in vitro evidence and taking into account the restricting clonal origin of human cancer cell lines, analysis of mutation of KiSS1 gene and of its expression would make sense in a preliminary cohort of resected breast carcinomas in order to assess its incidence and clinical significance.

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 [17–21]. Recently, brain metastases from breast and lung cancer were shown to have low KiSS1 mRNA and protein levels in comparison to the primary and the normal tissue [22, 23]. The molecular pathways through which KiSS1 exerts its antimetastatic effects are not yet elucidated [7–9, 24–26]. E-cadherin upregulation and reduction of matrix metalloprotease 9 (MMP9) expression, cytoskeletal reorganization, modulation of focal adhesion kinase activity, increased intracellular calcium release and inhibition of protein kinase C have been reported as effects of KiSS1 activation. Other groups observed KiSS1-induced PI3K-AKT pathway blockade resulting in induction of apoptosis, inhibition of CXCR4-mediated chemotaxis of neoplastic cells and reduction of NF-kB p50/p65 heterodimer formation.

In our study, we report for the first time the incidence and potential clinical significance of the single nucleotide substitution 242C>G in exon IVa of the KiSS1 gene. This nucleotide sequence alteration is the same described as 391C>G in the NCBI SNP Databank. The genetic change was restricted to the tumor in only 4% of patients as shown by its presence in the peripheral blood lymphocytes of 3 out of 5 patients bearing breast cancers with the substitution. The absence of an immunoreactive intact KiSS1 protein and the frequent, high-volume tumor involvement of axillary lymph nodes in patients harbouring tumors with the KiSS1 variant, though not reaching any statistical significance due to the small size of the patient cohort are preliminary observations that should be investigated further. Transfection experiments in neoplastic cell culture-based metastasis assays as well as in nude mice are needed in order to delineate the functional impact of the P81R substitution. No data exist at present on the incidence and clinical significance of the 242C>G germ-line polymorphism in a healthy population. KiSS1 is consitutively expessed in the placenta, hypothalamus/pituitary, lung, liver, breast and gastrointestinal tract. In view of the alteration of the tertiary structure of the protein incurred by the P81R substitution, its impact on the regulation of the neuroendocrine system, epithelial function and propensity for tumorigenesis in healthy subjects is worth exploring.

KiSS1 mRNA levels were detectable in only a third of breast carcinomas and did not correlate with the presence of KiSS1 exon IVa variation. The latter finding is expected in view of the primers used for KiSS1 mRNA detection: these were situated at the exon III/IV boundary and spanned a less than 100 base-long nucleic acid segment, 112 base pairs upwards from the point mutation identified. The lack of correlation of KiSS1 mRNA levels with tissue protein expression may also be interpreted in the same way. Both wild-type and altered mRNA were measured and molecular mechanisms that suppress transcription of the KiSS1 gene are probably independent from the 242C>G substitution. Possible mechanisms of KiSS1 down-regulation include homozygous deletion, promoter methylation and transcription factor deletion or inactivation [17, 19, 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 Sp1-binding sites of the gene`s promoter [27]. The AP2a transcription factor is encoded by a tumor-suppressor gene in chromosome 6p. Interestingly, chromosome 6 loss of heterozygosity has been associated with loss of KiSS1 expression and dissemination of gastric cancers and melanomas[28].

Several investigators reported inverse correlation of KiSS1 mRNA with tumor burden, depth of invasion, nodal or systemic metastases, stage and patient outcome in melanoma, thyroid cancer, bladder, ovarian cancer and esophagogastric cancer [17–22]. We were unable to establish any association of tumour KiSS1 mRNA levels with patient and tumor characteristics, neither any prognostic significance of KiSS1 mutational or transcriptional status for patient outcome. Still, the sample size of our cohort is inadequate for a powered analysis. In contrast to the cumulating published data suggesting an anti-metastatic function for KiSS1, 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 [29]. The authors conclude that KiSS1 is associated with poor prognosis and metastatic dissemination. 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, staining positively for KiSS1, while the antibody used is not specified. Finally, the possibility of mRNA detection of mutant inactive KiSS1 should also be considered. Indeed, as the authors state, decreased levels of KiSS1 receptor were associated with adverse outcome in the 124 patients with breast cancer.

To conclude, we report for the first time two distinct mechanisms of likely suppression of the anti-metastatic effect of KiSS1 in early breast cancer: a kisspeptin variant and suppression of KiSS1 transcription. Most, though not all, previously published preclinical and clinical evidence support the metastasis-suppressing role of the gene. If these data are validated and KiSS1 inactivation is confirmed to occur early in tumors at localized stages, clinical research towards development of MSG agonists or restoration of KiSS1 function will hold promise for arresting micrometastatic growth and preventing malignant relapse in cancer patients.

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