High tumoral levels of *Kiss1* and G-protein-coupled receptor 54 expression are correlated with poor prognosis of estrogen receptor-positive breast tumors

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Abstract

KiSS1 is a putative metastasis suppressor gene in melanoma and breast cancer-encoding kisspeptins, which are also described as neuroendocrine regulators of the gonadotropic axis. Negative as well as positive regulation of *KiSS1* gene expression by estradiol (E_2) has been reported in the hypothalamus. Estrogen receptor α (ERα level is recognized as a marker of breast cancer, raising the question of whether expression of *KiSS1* and its G-protein-coupled receptor (*GPR54*) is down- or upregulated by estrogens in breast cancer cells. *KiSS1* was found to be expressed in MDA-MB-231, MCF7, and T47D cell lines, but not in ZR75-1, L56Br, and MDA-MB-435 cells. *KiSS1* mRNA levels decreased significantly in ERα-negative MDA-MB-231 cells expressing recombinant ERα. In contrast, tamoxifen (TAM) treatment of ERα-positive MCF7 and T47D cells increased *KiSS1* and *GPR54* levels. The clinical relevance of this negative regulation of *KiSS1* and *GPR54* by E_2 was then studied in postmenopausal breast cancers. *KiSS1* mRNA increased with the grade of the breast tumors. ERα-positive invasive primary tumors expressed sevenfold lower *KiSS1* levels than ERα-negative tumors. Among ERα-positive breast tumors from postmenopausal women treated with TAM, high *KiSS1* combined with high *GPR54* mRNA tumoral levels was unexpectedly associated with shorter relapse-free survival (RFS) relative to tumors expressing low tumoral mRNA levels of both genes. The contradictory observation of putative metastasis inhibitor role of kisspeptins and RFS to TAM treatment suggests that evaluation of *KiSS1* and its receptor tumoral mRNA levels could be new interesting markers of the tumoral resistance to anti-estrogen treatment.

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Introduction

KiSS1 was first described as a gene encoding a protein involved in metastasis inhibition of melanoma and breast cancer (Lee *et al.* 1996, Lee & Welch 1997*a*,*b*). Loss of *KiSS1* expression has also been correlated with increased metastasis and/or cancer progression in malignant pheochromocytoma (Ohta *et al.* 2005), esophageal squamous cell carcinoma (Ikeguchi *et al.* 2004), bladder (Sanchez-Carbayo *et al.* 2003), ovarian (Jiang *et al.* 2005), gastric (Dhar *et al.* 2004), and pancreatic (Masui *et al.* 2004) tumors. In contrast, an increase in *KiSS1* expression with higher grade and metastatic capacity has

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been observed in breast tumors (Martin *et al.* 2005) and hepatocellular carcinoma (Ikeguchi *et al.* 2003). *KiSS1* encodes a 145-amino acid peptide that is further processed in the placenta to several truncated peptides of 10–54 residues called kisspeptins: these are the natural ligands of the G-protein-coupled receptor (GPR54; Kotani *et al.* 2001, Muir *et al.* 2001, Ohtaki *et al.* 2001, Bilban *et al.* 2004). GPR54-mediated anti-metastatic effects of Kp-54 (also known as metastin) and derived peptides have been reported in various cancer cell lines (Hori *et al.* 2001, Stafford *et al.* 2002, Masui *et al.* 2004, Becker *et al.* 2005).

The GPR54 pathway has also been implicated in the regulation of the hypothalamic–pituitary–gonadal axis. Functional integrity of GPR54 is indispensable for the normal function of the gonadotropic axis in humans (de Roux *et al.* 2003, Seminara *et al.* 2003, Lanfranco *et al.* 2005, Semple *et al.* 2005, Tenenbaum-Rakover *et al.* 2007) as well as mice (Funes *et al.* 2003, Seminara *et al.* 2003). *In vivo* administration of kisspeptin leads to a rapid increase in luteinizing hormone and follicle-stimulating hormone blood levels in humans (Dhillo *et al.* 2005), nonhuman primates (Plant *et al.* 2006), and rodents (Irwig *et al.* 2004, Navarro *et al.* 2005*a*,*b*, Patterson *et al.* 2006). Positive as well as negative regulation of *KiSS1* mRNA levels by steroid hormones has been reported in different nuclei of rat hypothalamus (Smith *et al.* 2005*a*,*b*). This regulation probably results from a direct effect of estradiol (E_2) as estrogen receptor α (ERα) is expressed within kisspeptin-immunoreactive cells present in the preoptic area and arcuate nucleus of the ovine hypothalamus (Franceschini *et al.* 2006).

A body of evidence supports the notion that ERα-mediated pathways play a critical role in breast carcinogenesis (Clarke *et al.* 2004). ERα level is consensually used as a prognostic marker of breast tumors and of the response to endocrine therapy (Clarke *et al.* 2004). In this study, we questioned whether *KiSS1* and/or *GPR54* expression is regulated by estrogen signaling pathways in breast cancer cell lines and whether this regulation may have clinical relevance in the evaluation of the tumoral response to tamoxifen (TAM) treatment.

Materials and methods

Cell lines and recombinant adenoviruses

All breast tumor cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and were maintained in red phenol-free Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Gaithersburg, MD, USA). All media were supplemented with 2 mM L-glutamine, and 10% fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin (Gibco). E_2 and TAM were purchased from Sigma–Aldrich.

All the adenovirus (Ad) vectors used in this study were non-replicative E1/E3-defective recombinant adenoviruses. AdCO1 was the control virus, carrying no insert. AdERα and AdERβ were previously described (Lazennec *et al.* 2001).

Optimal infection (MOI) was determined by staining for β-galactosidase activity after infection with AdLacZ as described previously (Randrianarison *et al.* 2001). For MDA-MB-231 cells, 8000 adenoviral particles per cell (pv/ml) were chosen as an optimal compromise between maximal efficiency of infection and minimal virus-induced cytopathic effect (data not shown).

Patients and samples

We analyzed samples of 92 primary breast tumors excised from women at our institution from 1980 to 1994. Tumor tissue samples of the 92 patients were collected in accordance with French regulations. Samples containing more than 70% tumor cells were considered suitable for this study.

The patients met the following criteria: primary unilateral non-metastatic postmenopausal invasive ductal carcinoma of the breast; ERα positivity (as determined at the protein level by biochemical methods (dextran-coated charcoal method until 1988 and enzymatic immunoassay thereafter) and confirmed by ERα real-time quantitative RT-PCR assay); complete clinical, histological, and biological information available; no radiotherapy or chemotherapy before surgery; and full follow-up at our institution. Standard prognostic factors are shown in Table 1. The patients had physical examinations and routine chest radiography every 3 months for 2 years, then annually. Mammograms were performed annually. The median follow-up was 7.7 years (range 1.5–15.0 y). All patients received postoperative adjuvant endocrine therapy (20 mg TAM daily for 3–5 years), and no other treatment; 27 patients relapsed. The first relapse events were distributed as follows: 23 metastases, and 4 local and/or regional recurrences with metastases.

To investigate the relationship between mRNA levels of *KiSS1* and *GPR54* and *ERα*-expression status, we also analyzed 36 additional primary breast tumors: 12 ERα negative and 24 ERα positive.

To investigate the relationship between mRNA levels of *KiSS1* and *GPR54* during breast cancer progression, we analyzed RNA pools of normal breast

Table 1 Characteristics of the 92 estrogen receptor α (ERα)-positive tumors from patients with and without relapse

	Number of patients	RFS	
		Number of events (%)[a]	*P* value[b]
Age			
≤70 y	46	15 (32.6)	NS
>70 y	46	12 (26.1)	
SBR histological grade			
I+II	78	19 (24.4)	0.04
III	14	8 (57.1)	
Lymph node status			
Negative	16	1 (6.3)	NS
Positive	76	26 (34.2)	
Macroscopic tumor size			
≤30 mm	70	15 (21.4)	0.0004
>30 mm	22	12 (54.4)	
Progesterone receptor			
Negative	27	12 (44.4)	NS
Positive	65	14 (21.5)	
RNA ERBB2 status[c]			
Overexpressed	12	5 (41.7)	NS
Normal	80	21 (26.3)	

[a]First relapse (local and/or regional occurrence, and/or distant metastases).
[b]Log-rank test. RFS, relapse-free survival; NS, not significant.
[c]See Bieche *et al.* (2001*b*).

tissue, benign breast tumors, ductal carcinoma *in situ*, ERα-positive invasive ductal grade I breast tumors, ERα-positive invasive ductal grade III breast tumors, and ERα-negative invasive ductal grade III breast tumors, prepared by mixing identical amounts of tumor RNA from five patients per group. Specimens of adjacent normal breast tissue from four breast cancer patients and normal breast tissue from three women undergoing cosmetic breast surgery were used as sources of normal RNA.

Western blot analysis

Nuclear protein extract (100 μg) was analyzed for steroid hormone receptor status by 10% Tris–glycine gel electrophoresis followed by western blot analysis.

Primary antibodies against ERβ (GR40) were purchased from Oncogene Research Products (Cambridge, UK), and antibodies against ERα (HC-20) and actin (I-19) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary peroxidase-conjugated goat anti-mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA), the donkey anti-rabbit IgG (Na934) was from Amersham Life Sciences, and the donkey anti-goat IgG was from Santa Cruz Biotechnology.

Immunofluorescence staining

Twenty-four hours after plating on polylysine-coated slides, cells were fixed with 3.7% paraformaldehyde in PBS for 15 min, and rinsed three times with PBS. They were then incubated in PBS containing 2% BSA for 1 h, permeabilized with 0.2% Triton X-100, and then incubated with an anti-Kp-10 polyclonal rabbit antibody (1/200) for 1 h (Franceschini *et al.* 2006). After washing, cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Slides were analyzed under a Leica fluorescence microscope.

Reverse transcription

Total RNA was isolated from cultured cells or human tissues with TRIZOL (Invitrogen) and quantified by u.v. 260/280 nm absorption ratio. The quality of the RNA samples was determined by 1% agarose gel. Total RNA (1 μg) was reverse transcribed in a final volume of 20 μl using MuLvRT (Perkin–Elmer, Waltham, MA, USA) as per the manufacturer's instructions. Negative controls (1 μg RNA sample) were included in each PCR to exclude genomic DNA contamination.

Semiquantitative RT-PCR

Semiquantitative reverse transcriptase (RT) PCR was used to screen a number of human breast cancer cell lines. cDNA was used as the template for PCR and the reaction mixture contained 5% DMSO. The thermal cycling conditions comprised one cycle for 2 min at 94 °C, 25–40 cycles at 94 °C for 45 s, 60 °C, or 55 °C (ERα and β-actin respectively) for 45 s, and 72 °C for 30 s. The PCR products were resolved on 3% low-melting agarose gels. Amplification of the β-actin fragment was used for RT normalization. The primer pairs are given in Table 2.

Real-time RT-PCR

Breast tumor cell lines

To investigate the transcriptional regulation of *KiSS1* and *GPR54* expression by estrogen pathways, we analyzed breast tumor cell lines by quantitative PCR using the fluorescent Taqman methodology. Oligonucleotide primers and Taqman probes for *KiSS1* and *GPR54* genes were as previously described (Ohtaki *et al.* 2001).

All PCRs were performed using the ABI Prism 7700 Sequence Detection System (Perkin–Elmer Applied Biosystems) under the conditions recommended by the manufacturer. Briefly, the thermal cycling conditions

Table 2 Primer pairs used for RT-PCR amplification of transcripts in breast tumor cells

Gene		Primer sequences
ERα	F	5′-CAGGCTACCATTATGGAGT-3′
	R	5′-CCAGGCTGTTCTTCTTAGAG-3′
ERβ	F	5′-GGTCCATCGCCAGTTATCAC-3′
	R	5′-GGAGCCACACTTCACCATTC-3′
KiSS1	1F[a]	5′-GGACCTGCCTCTTCTCACCA-3′
	1R[a]	5′-ATTCTAGCTGCTGGCCTGTG-3′
	2F[a]	5′-TTCTAGACCCACAGGCCAGCA-3′
	2R[a]	5′-GACGGCTCAGCCTGGCAGT-3′
GPR54	1F[a]	5′-AACTTCTACATCGCCAACCTG -3′
	1R[a]	5′-CACCGAGACCTGCTGGATGTA-3′
	2F[a]	5′-CGACTTCATGTGCAAGTTCGTC-3′
	2R[a]	5′-CACACTCATGGCGGTCAGAG-3′
β-Actin	F	5′-GAAGCATTTGCGGTGGACCAT-3′
	R	5′-TCCTGTGGCATCCACCAAACT-3′

F, forward; R, reverse.
[a]Primers used in SYBR green methodology (see Materials and methods).

comprised an initial denaturation step at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Experiments were performed with duplicates for each data point.

We compared the *KiSS1* and *GPR54* mRNA levels of breast tumor cell lines with a human placenta sample as a positive control. Final results were calculated using the equation $2(\Delta C_t$ sample–ΔC_t calibrator), where ΔC_t values of the sample and calibrator are determined by subtracting the average C_t value of the target gene from the average C_t value of RNA 18S (internal control). Primers for *18S*were from Perkin–Elmer Applied Biosystems. Each sample was normalized such that the quantitative values corresponding to the placenta sample equaled 1.00.

Breast tumors

To investigate the quantitative relationship between *KiSS1* and *GPR54* mRNA levels and *ERα* expression during breast cancer progression, we analyzed normal breast tissue and primary breast tumors by quantitative PCR using the fluorescent SYBR green methodology. RNA extraction, cDNA synthesis, and PCR conditions were as in Bieche *et al.* (2001*a*). Briefly, the thermal cycling conditions comprised an initial denaturation step at 95 °C for 10 min, 50 cycles at 95 °C for 15 s, and 65 °C for 1 min. Each sample was normalized on the basis of two endogenous RNA control genes involved in two cellular metabolic pathways, namely TATA-binding protein (TBP; Genbank accession NM_003194) and Ribosomal Protein Large P0 (RPLP0; Genbank accession NM_001002). Primers for *TBP*, *RPLP0*, and *ERα* genes were as described previously (Bieche *et al.* 1999, 2001*a*).

Results expressed as *N*-fold differences in target gene expression relative to the *TBP* (or *RPLP0*) gene, and termed N_{target}, were determined as $N_{target} = 2^{\Delta C_t sample}$, where the ΔC_t value of the sample is determined by subtracting the average C_t value of the target gene from the average C_t value of the TBP (or *RPLP0*) gene (Bieche *et al.* 1999, 2001*a*).

Statistical analysis

As the mRNA levels did not fit a Gaussian distribution, a) the mRNA levels in each subgroup of samples were characterized by their median values and ranges, rather than their mean values and coefficients of variation, and b) relationships between the molecular markers, and clinical and biological parameters were tested by nonparametric Mann–Whitney *U* test (Mann & Whitney 1947). Differences between two populations were judged significant at confidence levels greater than 95% ($P < 0.05$).

To visualize the capacity of a given molecular marker to discriminate between two populations, in the absence of an arbitrary cutoff value, we summarized the data in a ROC (receiver operating characteristics) curve (Hanley & McNeil 1982). These curves plot sensitivity (true positives) on the *y*-axis against 1-specificity (false positives) on the *x*-axis, considering each value as a possible cutoff. The area under the curve (AUC) was calculated as a single measure of the discriminatory capacity of each molecular marker. When a molecular marker has no discriminative value, the ROC curve lies close to the diagonal and the AUC is close to 0.5. When a marker has strong discriminative value, the ROC curve moves to the upper left-hand corner (or to the lower right-hand corner), and the AUC is close to 1.0 (or 0).

Relapse-free survival (RFS) was determined as the time interval between diagnosis and detection of the first relapse (local and/or regional recurrence, and/or distant metastasis). Survival distributions were estimated by the Kaplan–Meier method (Kaplan & Meier 1958), and the significance of differences between survival rates was ascertained using the log-rank test (Peto *et al.* 1977).

Results

Expression of KiSS1 and GPR54 in breast tumor cell lines

KiSS1 and *GPR54* mRNA status was studied in six breast tumor cell lines (T47D, MCF7, ZR75-1, MDA-MB-231, MDA-MB-435, and L56Br; Fig. 1). Semi-quantitative RT-PCR performed with two primers,

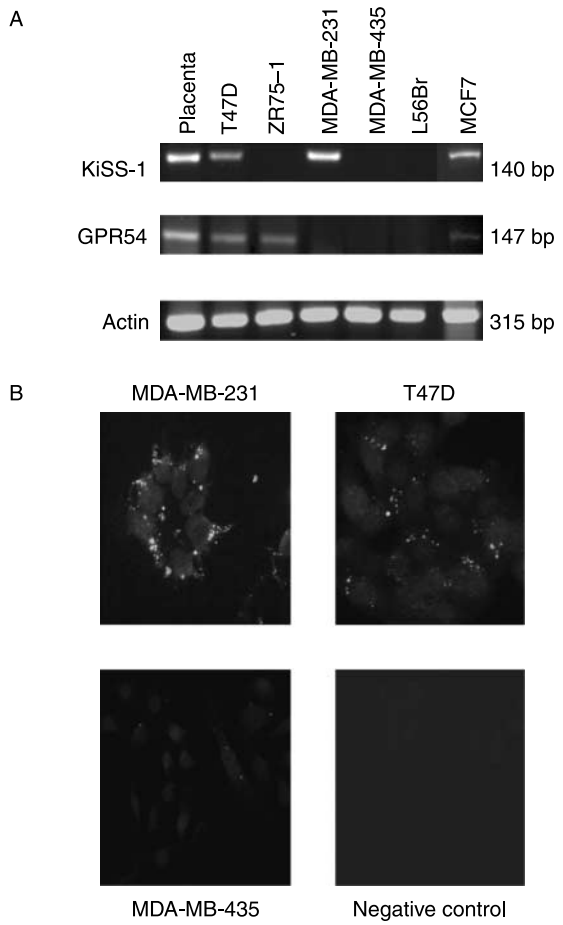

Figure 1 Expression of KiSS1 and GPR54 in breast tumor cell lines. (A) RT-PCR amplification conditions are described in Materials and methods. Semiquantitative PCR products were analyzed by 3% low-melting agarose gel electrophoresis. B. MDA-MB-231, T47D, and MDA-MB-435 breast tumor cells were fixed and stained with an anti-metastin antibody as described in Materials and methods.

located within exon 2 and at the junction between exons 2 and 3, showed high *KiSS1* mRNA levels in MDA-MB-231, similar to those observed in the human placenta sample (Fig. 1A). MCF7 and T47D cells expressed lower *KiSS1* mRNA levels than MDA-MB-231 cells. No expression of *KiSS1* was detected in ZR75-1 or L56Br cells. Our results confirmed the absence of *KiSS1* expression in MDA-MB-435 cells, as previously reported (Lee *et al.* 1996, Lee & Welch 1997*b*, Ohtaki *et al.* 2001). *GPR54* mRNA was found in MCF7, T47D, and ZR75-1 cells at lower levels than in the placenta. We failed to detect *GPR54* mRNA in L56Br, MDA-MB-231, or MDA-MB-435 cells. The *KiSS1* expression results were confirmed using two other primers (KiSS1-2F and KiSS1-2R). The *KiSS1* gene expression found in MDA-MB-231 cells was in agreement with Yan *et al.* (2001), but not with reports from Martin *et al.* (2005) and Mitchell *et al.* (2006). This discrepancy may be explained by primer design and PCR conditions or may reflect clonal divergence in these cells from one laboratory to the next.

Immunofluorescence staining performed with an antibody against amino acid residues 45–54 of human metastin showed lower labeling in T47D than in MDA-MB-231 cells, and no labeling in MDA-MB-435 cells (Fig. 1B), confirming the RT-PCR results.

Estrogen-induced downregulation of *KiSS1* gene expression in MDA-MB-231 cells expressing ERα or ERβ

ERα and ERβ protein levels were detected in T47D and ZR75-1 breast tumor cells (Fig. 2). In contrast, MDA-MB-231 and MDA-MB-435 cells showed no ERα or ERβ expression. These latter two cell lines expressed neither the progesterone receptor nor the androgen receptor (data not shown).

To study the effects of E_2 on *KiSS1* gene expression, ERα-negative MDA-MB-231 cells were infected with recombinant adenoviruses encoding ER α (AdERα and β AdERβ) (Fig. 3A). Twenty-four hours postinfection, *KiSS1* mRNA level was determined by real-time RT-PCR. ERα-negative MDA-MB-231 cells expressed a high level of *KiSS1* mRNA (Fig. 3B). However, 12 h after addition of 10^{-7} M E_2 to the medium, we observed a significant decrease in *KiSS1* mRNA levels of AdERα- and AdERβ-infected MDA-MB-231 cells, by 3.3 ± 0.45- and 4.6 ± 0.45-fold respectively, relative to cells infected with control adenovirus (AdCO1) only ($P < 0.005$). In contrast, the addition of E_2 alone or the

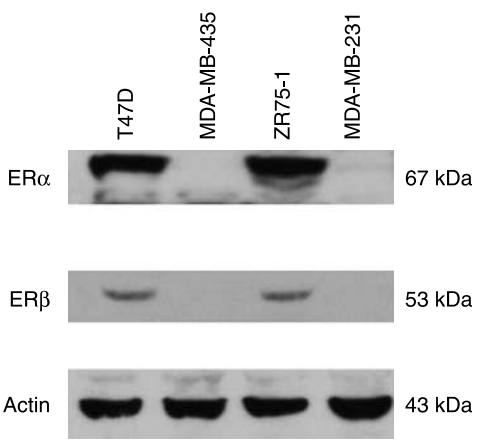

Figure 2 Expression of ERα and ERβ in breast tumor cell lines. Nuclear cell extracts (100 μg) were separated by 10% tris–glycine gel electrophoresis, transferred to a nitrocellulose filter and immunoblotted for ERα, ERβ and β-actin as described in Marot *et al.* (2006).

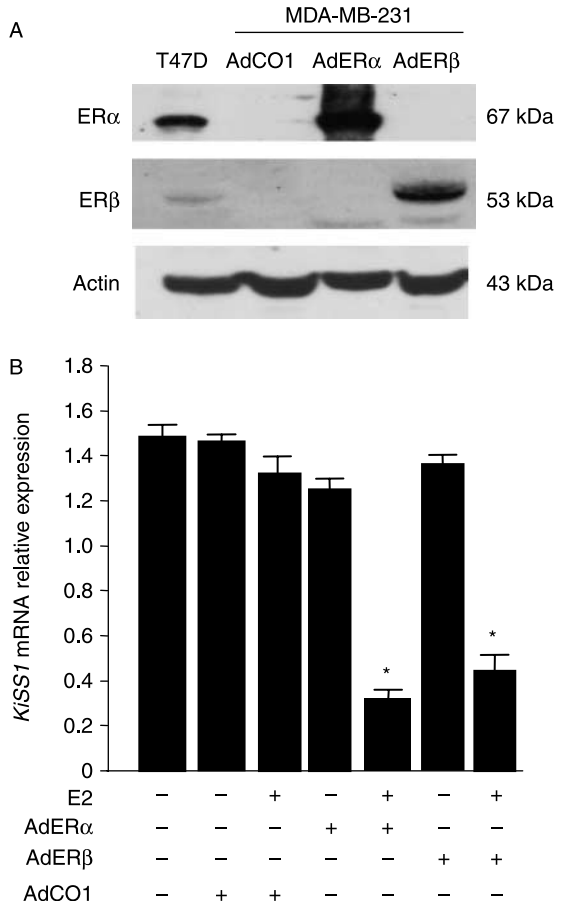

Figure 3 Effect of ERα and ERβ in MDA-MB-231 (ERα-deficient) breast tumor cells. MDA-MB-231 cells were infected with AdCO1, AdERα and AdERβ at a MOI of 8000 pv/cell. After 12 h, DMEM was supplemented with 10^{-7}M E_2. (A) Nuclear cell extracts were subjected to immunoblotting using anti-ERα, anti-ERβ, and anti-β-actin antibodies (Marot *et al.* 2006). T47D cells were used as positive controls of ERα and ERβ expression. (B) Twenty-four-hour postinfection, KiSS1 mRNA levels in MDA-MB-231 cells were determined by real-time RT-PCR. KiSS1 mRNA levels are expressed relative to placental levels set to 1. Transcript values are representative of at least two independent experiments.

reintroduction of ERα or ERβ without E_2 did not change *KiSS1* transcript levels.

TAM upregulation of *KiSS1* and *GPR54* mRNA expression in ERα-positive cells

As reintroduction of ER in the presence of E_2 induced negative regulation of *KiSS1*, we tested whether functional inhibition of ER would induce an increase in *KiSS1* gene expression in ER-expressing cells. T47D, MCF7, and ZR75-1 ER-positive cells were cultured in phenol red-free DMEM medium without (control) or with the synthetic anti-estrogen TAM (10^{-6} M) for 24 h. TAM induced a three- and twofold increase in *KiSS1* expression in MCF7 and T47D cells respectively (Fig. 4A; $P<0.05$). It is worth noting that in ZR75-1 cells, *KiSS1* levels after the addition of TAM remained undetectable. *GPR54* mRNA levels in Tam-treated MCF7, T47D, and ZR75-1 cells increased 2.5-, 3.4-, and 7.3-fold respectively (Fig. 4B; $P<0.05$).

Increase in *KiSS1* mRNA levels with breast tumor grade

Levels of *KiSS1* and *GPR54*, as well as of the gene *MKI67* that encodes the proliferation-related antigen Ki-67, were studied in a set of five breast tumor pools relative to normal breast tissue (Table 3). Pools of normal breast tissues, benign breast tumors, ductal carcinoma *in situ*, ERα-positive invasive ductal grade I breast tumors, ERα-positive invasive ductal grade III

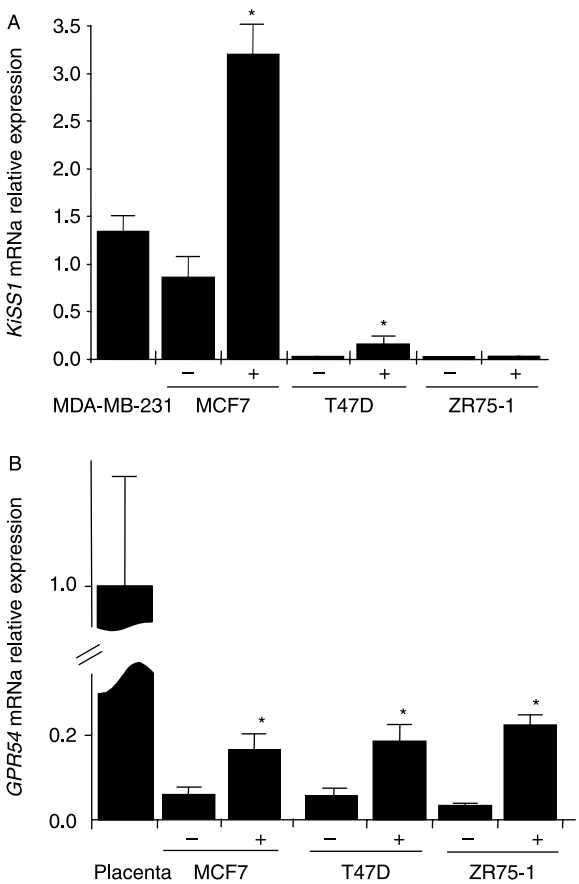

Figure 4 Effect of tamoxifen on KiSS1 and GPR54 mRNA expression in ER-positive breast tumorcells. T47D, ZR75-1, and MCF-7 cells were incubated in phenol red-free DMEM without (−) or with (+) 1 μM tamoxifen for 24 h and total RNA was isolated. KiSS1 and GPR54 mRNA levels were determined by real-time RT-PCR using Taqman methodology as described in Materials and Methods. *Significant difference ($P<0.05$).

Table 3 *KiSS1*, G-protein-coupled receptor (*GPR54,*) and *Ki-67* mRNA expression during breast cancer progression. Total RNA from pooled breast tumor samples was reverse transcribed and analyzed by quantitative PCR (SYBR green methodology, see Materials and methods). mRNA levels are relative to normal breast tissue pool (1.0 arbitrary units)

	KiSS1	*GPR54*	*Ki-67*
Benign tumors	2.5	0.68	5.2
Ductal carcinoma *in situ*	2.7	0.23	17.5
ERα-positive invasive ductal tumors			
Grade I	5.9	31.9	14.4
Grade III	11.1	31.2	29.4
ERα-negative invasive ductal Grade III tumors	56.8	16.5	27.7

breast tumors, and ERα-negative invasive ductal grade III breast tumors were each prepared by mixing identical amounts of tumor RNA from five patients.

Relative to normal mammary tissue, levels of *KiSS1* mRNA were higher in benign tumors and ductal carcinoma *in situ*(∼2.5-fold). No difference was observed for *GPR54* mRNA levels. For grade III invasive ductal breasttumor pools, higher *KiSS1* mRNA levels were measured in ERα-negative tumors than in ERα-positive ones (∼56- and 11-fold higher than in normal tissue respectively). A high level of *GPR54* mRNA was also detected in invasive tumors, although this level was twofold higher in ERα-positive than in ERα-negative breast tumors (∼31- and 16-fold higher than in normal tissue respectively). *MKI67* level increased with the grade of the tumors but no difference was observed between ERα-negative and ERα-positive tumors. Immunohistochemistry analysis with a polyclonal antibody against the C-terminal end of Kp54 has confirmed that tumors expressing high *KiSS1* mRNA levels also expressed metastin as a peptide (data not shown).

mRNA expression of *KiSS1* and *GPR54* in 24 ERα-positive breast tumors and 12 ERα-negative breast tumors

To further investigate quantitative relationships between mRNA levels of *KiSS1*, *GPR54*, and *ERα*, we analyzed 36 primary breast tumors: 12 ERα negative and 24 ERα positive. The median level of *ERα* mRNA was set at 1 (arbitrary units; range 0.2–5.1) in the ERα-negative breast tumor group and 711 (range 70.8–1938) in the ERα-positive breast tumor group ($P=0.0\,000\,014$). *KiSS1* mRNA level was significantly lower (approximately sevenfold) in the 24 ERα-positive breast tumors when compared with the 12 ERα-negative breast tumors, while *GPR54* mRNA was slightly (but not significantly) higher (∼3.7-fold) in the former versus the latter (Table 4).

KiSS1 and GPR54 mRNA levels in breast tumors from postmenopausal patients treated with primary surgery and adjuvant TAM

mRNA levels of *KiSS1* and *GPR54* were determined by real-time RT-PCR in a cohort of 27 ERα-positive breast tumor patients who relapsed and 65 ERα-positive breast tumor patients who did not (Table 1). All 92 ERα-positive tumors were from postmenopausal patients treated with primary surgery followed by TAM adjuvant treatment. In this series of tumors, we found no correlation between *KiSS1* and *GPR54* mRNA levels ($r=+0.094$, $P=0.38$; Spearman's rank correlation test).

For univariate analysis (log-rank test), the 92 ERα-positive breast tumors were divided into two equal subgroups of 46 tumors based on mRNA levels of *KiSS1* (or *GPR54*). The 'high' subgroup was composed of the 46 tumors with the highest mRNA level and the 'low' subgroup was composed of the 46 tumors with the lowest mRNA level. This analysis showed that high tumoral level of *KiSS1* mRNA is significantly correlated with shorter *RFS ($P=0.039$; Fig. 5A). The outcomes of the 46 patients with high *KiSS1* mRNA levels (8-year RFS 56.1%±8.6) were significantly worse than those of the 46 patients with low *KiSS1* mRNA levels (8-year RFS 82.6%±6.0). We also observed a trend (albeit not significant) towards a linkage between high *GPR54* mRNA levels and shorter RFS ($P=0.1$).

Table 4 Statistical analyses of *KiSS1* and G-protein-coupled receptor (*GPR54*) expressions in estrogen receptor α (ERα)-positive breast tumors relative to ERα-negative breast tumors. Total RNA was reverse transcribed and analyzed by quantitative PCR. mRNA levels are relative to levels in ERα-negative breast tumors (1.0 arbitrary units)

	ERα-negative ($n=12$)	**ERα-positive** ($n=24$)	***P*** [a]	**ROC-AUC** [b]
KiSS1	1.0 (0.24–2.96)	0.14 (0.01–8.96)	0.00006	0.083
GPR54	1.0 (0.11–15.3)	3.71 (0.03–53.8)	NS	0.700
ERα	1.0 (0.2–5.1)	711 (70.8–1938)	0.0000014	1.000

[a]Log-rank test: NS, not significant.
[b]Scarff Bloom Richardson classification: ROC-AUC, receiver operating characteristics-area under curve.

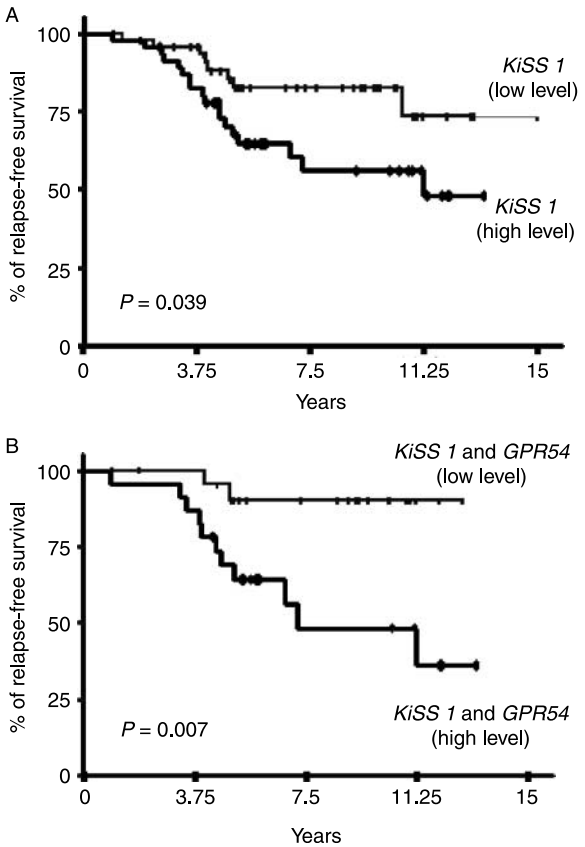

Figure 5 Correlation between KiSS1 and GPR54 mRNA tumoral levels and relapse-free survival (RFS). (A) RFS curves for breast tumors with low KiSS1 gene expression (blue line) and high *KiSS1* gene expression (black line). (B) RFS curves for breast tumors with both low KiSS1 and low *GPR54* gene expression (blue line), and both high KiSS1 and high *GPR54* gene expression (black line).

Two unrelated molecular markers may provide a more accurate prediction of hormone responsiveness when considered together rather than individually. As *KiSS1* expression was not related to *GPR54* expression in this series of 92 tumors, and both *KiSS1* and *GPR54* mRNA tumoral levels were linked or tended towards linkage to RFS, we combined *KiSS1* and *GPR54* statuses to identify four separate prognostic subgroups (23 patients in each) with significantly different RFS curves. The patients with the poorest prognosis had high *KiSS1* mRNA levels and high *GPR54* mRNA levels (high–high subgroup, 8-year RFS 48.3%$\pm$12.4), while those with the best prognosis had low *KiSS1* mRNA levels and low *GPR54* mRNA levels (low–low subgroup, 8-year RFS 90.2%$\pm$6.6) ($P=0.007$; Fig. 5B). The other two subgroups, low *KiSS1*/high *GPR54* (8-year RFS 75.1%$\pm$9.8) and high *KiSS1*/low *GPR54* (8-year RFS 67.2%$\pm$10.4), exhibited intermediate outcomes.

Discussion

KiSS1 is a human tumor metastasis suppressor gene with a specific role in breast and melanoma tumor development (Lee *et al.* 1996, Lee & Welch 1997*b*, Ohtaki *et al.* 2001). We investigated the hypothesis that *KiSS1* expression is regulated by estrogen signaling pathways in breast tumors by following two arguments: differential regulation of *KiSS1* hypothalamic levels has been observed in ovariectomized rats after E_2 administration (Smith *et al.* 2005*a*,*b*) and ERα is considered an important prognostic factor for breast tumors.

To study the complex expression of *KiSS1* and *GPR54*, we used several primer pairs and PCR methodologies (SYBR green and Taqman) with different housekeeping genes. Three breast tumor cell lines (ERα-negative MDA-MB-231 and ERα-positive T47D and MCF7) expressed high and low *KiSS1* mRNA levels when compared with a placenta sample. We also observed low levels of *GPR54* mRNA in three ERα-positive cell lines, MCF7, T47D, and ZR75-1.

In this study, we showed regulation of the expressions of *KiSS1* and its receptor through estrogen signaling pathways in breast tumor cell lines. We reported a significant E_2-induced decrease in *KiSS1* mRNA level in AdERα- and AdERβ-infected ER-negative MDA-MB-231 cells when compared with Ad control-infected cells. Interestingly, the contribution of ERβ in ER-deficient breast tumor cells is as effective as the reintroduction of ERα. Conversely, TAM administration upregulated *KiSS1* and *GPR54* expression in ER-positive breast tumor cells. These findings are in agreement with previous data in female rodents in which E_2 treatment reversed the increase in hypothalamic *KiSS1* mRNA level induced by ovariectomy (Navarro *et al.* 2004, Smith *et al.* 2005*a*). However, in contrast to our results showing similar downregulation by ERα and ERβ, selective ERβ agonist did not prevent the hypothalamic *KiSS1* mRNA increase after ovariectomy (Navarro *et al.* 2004). Recently, activator protein 2 (AP-2α has been described as a possible positive transcriptional regulator of *KiSS1* in breast cancer cell lines via interaction with specificity 1 protein (Sp1; Mitchell *et al.* 2006). *AP-2α* mRNA level is downregulated by estrogens in breast tumors (Orso *et al.* 2004), suggesting that the effect of E_2 on *KiSS1* expression may be mediated by a decrease in *AP-2α* level. Cofactor required for Sp1-3 (CRSP3) and TXNIP (thioredoxin-interacting protein) are also candidate transcription factors for upregulation of *KiSS1* expression in melanoma (Goldberg *et al.* 2003). These findings converge with our results to indicate that ER-mediated regulation of *KiSS1* transcription

predominates in the complex transcriptional context of mammary tumor progression.

These results are of primary clinical importance for correlating *KiSS1* expression levels with breast tumor progression. *KiSS1* expression was analyzed in a series of primary breast tumors according to histological grade and ERα status. High levels of *KiSS1* were detected in breast tumors but not in normal mammary tissues. *KiSS1* level was high in the first stages of the disease and increased with tumor progression, whereas *GPR54* expression increased in invasive ductal tumors but not in benign tumors or ductal carcinoma *in situ*. The correlation between *KiSS1* levels and tumor progression confirmed the results reported by Martin *et al.* (2005). The rise in *GPR54* appears to correlate better with metastatic capacity than with tumor growth, suggesting that the control of invasive properties in breast tumors requires simultaneous overexpression of *KiSS1* and its receptor. A similar increase in *GPR54* mRNA levels has been observed in malignant tumors when comparing renal cell carcinoma with adjacent normal tissue (Lenburg *et al.* 2003). There are similarities in the behaviors of invasive trophoblasts and invasive breast cancer cells (Murray & Lessey 1999). Our results are thus in agreement with the proposed role for *KiSS1*/*GPR54* autocrine and/or paracrine signaling pathways in trophoblast cells during gestation (Bilban *et al.* 2004, Terao *et al.* 2004). *KiSS1* appears to be a molecular marker for human breast tumors and *GPR54* a marker of invasive-grade tumors.

Despite the high *KiSS1* mRNA levels in breast tumors relative to normal breast tissue, *KiSS1* expression was negatively associated with ERα status in these tumors. We also observed a high variability of *KiSS1* expression in ERα-positive tumors, suggesting that *KiSS1* expression level appeared to be an attractive molecular marker for predicting TAM responsiveness of ERα-positive postmenopausal breast cancers. In ER-positive tumors, high *KiSS1* and *GPR54* mRNA levels were significantly associated with shorter RFS for postmenopausal women with unilateral invasive primary breast tumors: the outcome for patients with high *KiSS1* and *GPR54* levels was worse than for those with low KiSS1 and GPR54 levels. Although KiSS1 mRNA level (as well as the combination *KiSS1* and *GPR54* mRNA level) was found to be a significant predictor of patient outcome in univariate analyses, when adjusting for known prognostic markers such as SBR histological grade and macroscopic tumor size (Table 1) using multivariate Cox analyses, *KiSS1* mRNA level (and the combination *KISS1*/*GPR54*) was no longer significantly associated with RFS. This finding may be due, in part, to our small sample size of patients. A large sample series are needed to assess whether KISS1 and/or the combination KISS1/GPR54 are independent markers for RFS in breast cancer.

Considering that *KiSS1* was first described as an inhibitor of metastasis, the poor evolution of ERα-positive tumors with high *KiSS1*levels was unexpected. The high tumoral *KiSS1* and *GPR54* levels detected in patients with short RFS may be a normal response to the high metastatic potential of these tumoral cells. In this case, decreases in *KiSS1* and *GPR54* tumoral expression may lead to metastasis. A reduction in metastasis suppressor gene expression has recently been reported in breast cancer brain metastases (Stark *et al.* 2005). It would thus be interesting to compare *KiSS1* and *GPR54* expression levels in brain, liver, or bone metastases from 'high–high' tumors with *KiSS1* expression in metastases resulting from 'low–low' tumors. This high *KiSS1* expression in ERα-positive breast tumor cells with poor prognosis may also reflect hormonal resistance to E_2, impeding the beneficial action of TAM treatment in these patients. Intratumoral somatic mutations leading to defects in KiSS1 maturation is unlikely as high metastin labeling has been observed with a specific antibody in tumors expressing high KiSS1 mRNA level. Alternatively, functional inactivation of GPR54 or defects in intracellular pathways stimulated by GPR54 might explain these high levels of *GPR54* and *KiSS1* expression in tumors with poor prognosis. Although a possible prometastatic effect of *KiSS1* cannot be excluded, in such a hypothesis, adjuvant treatment blocking estrogen signaling pathways might be deleterious in 'low–low' and 'high–high' tumors.

These data show that *KiSS1*and *GPR54* are estrogen-regulated genes, and their expression levels must therefore be analyzed relative to ERα status. *KiSS1* level appears to be an interesting new marker for distinguishing breast tumors from normal mammary tissues. Resistance to anti-estrogens is one of the major challenges in the treatment of ER-positive breast tumors (Osborne 1998). In addition, with gene-expression assays recently proposed to predict tumoral responses to TAM (Paik *et al.* 2004, 2006, Jansen *et al.* 2005), our data indicate that an evaluation of *KiSS1* and its receptor's tumoral mRNA levels could be important for the clinical management of breast tumors.

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