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RESEARCH ARTICLE

KiSS-1 methylation and protein expression patterns contribute to diagnostic and prognostic assessments in tissue specimens for colorectal cancer

Patricia Moya · Sergio Esteban · Antonio Fernandez-Suarez · Marisa Maestro · Manuel Morente · Marta Sánchez-Carbayo

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Abstract *KISS1* is a metastasis suppressor lost in several solid malignancies. We evaluated the clinical relevance of KiSS-1 methylation and its protein expression in colorectal cancer. The epigenetic silencing of KiSS-1 by hypermethylation was tested in colon cancer cells (n=5) before and after azacytidine treatment. KiSS-1 methylation was evaluated by methylation-specific PCR in colorectal cancer cells, and normal, benign, and tumor tissues (n=352) were grouped in a training set (n=62) and two independent validation cohorts (n=100 and n=190). KiSS-1 protein expression was analyzed by immunohistochemistry on tissue arrays. KiSS-1 hypermethylation correlated with transcript and protein expression loss, being increased in vitro by azacytidine. Methylation rates were 53.1, 70.0, and 80.0 % in the training and validation sets, respectively. In the training set, KiSS-1 methylation rendered a diagnostic accuracy of 72.7 % (p=0.002). Combination of KiSS-1 methylation and serum CEA (p=0.001) increased the prognostic utility of CEA alone (p=0.022). In the first validation set, KiSS-1 methylation correlated with tumor grade (p=0.011), predicted recurrence (p=0.009), metastasis (p=0.004), disease-free (p=0.034), and overall survival (p=0.015). In the second validation cohort, KiSS-1 methylation predicted disease-specific survival (p=0.030). In the training set, cytoplasmic KiSS-1 expression was significantly higher in nonneoplastic biopsies as compared to colorectal tumors (p<0.0005). In the validation set, loss of cytoplasmic expression correlated with tumor stage (p=0.007), grade (p=0.035), recurrence (p=0.017), and disease-specific survival (p=0.022). KiSS-1 was revealed epigenetically modified in colorectal cancer. The diagnostic and prognostic utility of KiSS-1 methylation and expression patterns suggests their assessment for the clinical management of colorectal cancer patients.

Keywords Colorectal cancer · KiSS-1 · Methylation · Biomarker · Tissue array · Immunohistochemistry

P. Moya · S. Esteban · M. Sánchez-Carbayo
Tumor Markers Group, Molecular Pathology Program,
Spanish National Cancer Center,
Madrid, Spain

A. Fernandez-Suarez
Biochemistry Department, Hospital Alto Guadalquivir,
Andujar, Jaén, Spain

M. Maestro
Biochemistry Department, Hospital Clinico Universitario,
Madrid, Spain

M. Morente
Tumor Bank Unit, Molecular Pathology Program,
Spanish National Cancer Center,
Madrid, Spain

M. Sánchez-Carbayo (✉)
Tumor Markers Group, 310A,
Spanish National Cancer Research Center,
Melchor Fernandez Almagro 3,
28029, Madrid, Spain
e-mail: mscarbayo@cnio.es

Introduction

Colorectal cancer (CRC) is one of the most frequent and aggressive solid tumors [1, 2]. Colorectal tumors arise as a consequence of the accumulation of genetic (e.g., gene amplification or mutations) and epigenetic alterations (e.g.,

aberrant DNA methylation, chromatin modifications or microRNAs), among others, in colon epithelial cells during neoplastic transformation [3–6]. Transcriptional inactivation by promoter hypermethylation of critical cancer-related genes is recognized as the most common epigenetic alteration in colorectal tumors [7, 8]. Substantial efforts have been made to determine the role of aberrant DNA methylation in colorectal carcinogenesis, and the biomarker utility of methylation patterns for the clinical management of CRC patients.

KISS1 was identified to suppress metastases in melanoma and breast cancer cells [9–15]. Being expressed in the central nervous system, testis, ovary, pancreas, and bladder, its highest concentrations were found in the placenta [13–16]. Mapping to 1q32 [16], *KISS1* encodes a 145-amino acid protein, being processed into kisspeptins of several sizes [17–19]. Kisspeptins are physiologically functional at controlling the onset of puberty and at inhibiting cancer metastasis of different tumor types [9–16]. Associations between *KISS1* expression loss and increased tumor progression and poor prognosis were found in several solid tumors [16–26].

KiSS-1 was recently shown epigenetically silenced by hypermethylation in bladder cancer [27]. KiSS-1 methylation was clinically useful as a tumor stratification biomarker and clinical outcome prognosticator for bladder cancer patients [27]. In this report, we evaluated whether KiSS-1 expression and methylation patterns differed in CRC, and their potential clinical relevance. The novelty of our findings is high, because (a) KiSS-1 was not previously described in CRC and (b) the methylation and differential expression KiSS-1 patterns provided a diagnostic and prognostic utility in tissue specimens for the clinical management of CRC patients.

Materials and methods

Tumor samples Primary colorectal tissues were collected following the protection guidelines of human subjects at participating hospitals after written consent approval and after the Institutional Review Board (IRB) approved the protocols at collaborating institutions. Inclusion criteria of newly diagnosed CRC patients were based on the histopathologic information, covering from early to advanced stages. It was also required to have tissue material available for obtaining high-quality DNA for methylation analyses. The initial training set included paraffin blocks of benign intestine disease (n=31) and colorectal tumors (n=31), for which matching normal intestine was available. This set served to (a) screen methylation rates in matching pairs of normal and colorectal tumors, and in benign intestinal disease; (b) evaluate the association of KiSS-1 expression with its methylation status; and (c) evaluate the association of methylation and expression with clinicopathologic variables. Demographic information of CRC patients indicated 23 males and eight females, with a median age of 68.2 years (range, 38.0–92.0 years). In control individuals without CRC, there were 24 males and seven females, with a median age of 61.6 years (range, 40.0–82.0 years). Two independent validation sets were analyzed. The first validation cohort included 100 clinical stage II frozen colorectal tumors. This set served to validate (a) methylation rates and (b) associations of methylation with clinicopathologic variables. Demographic information of CRC patients indicated 54 males and 46 females, with a median age of 72.3 years (range, 35.0–92.0 years).The second validation cohort included a series of 190 paraffin blocks. This set served to validate (a) methylation rates, (b) association of KiSS-1 expression with its methylation status, and (c) association of methylation and expression with clinicopathologic variables. Histopathologic staging of colorectal tumors was defined under standard criteria after subsequent surgical interventions [28]. Demographic information of CRC patients indicated 96 males and 94 females, with a median age of 69.0 years (range, 36.0–84.0 years). Distribution of clinical variables is provided in Table 1.

Methylation analyses of the promoter of KiSS-1 The methylation status of KiSS-1 (Fig. 1a) was analyzed by two PCR analysis strategies of bisulfite-modified genomic DNA, which induces chemical conversion of unmethylated, but not methylated, cytosine to uracil. First, genomic sequencing of both DNA strands of *KiSS-1* was analyzed after bisulfite treatment, of at least three clones per cancer cell line analyzed [27]. Confirmation on at least two independent clones was required to assign methylation sites. The second strategy used PCR with primers specific for either the methylated or the modified unmethylated DNA [methylation-specific PCR (MS-PCR)] [27]. OCT and paraffin-embedded tissues were macro-dissected based on hematoxylin–eosin evaluations ensuring a minimum of 75 % of tumor cells before DNA extraction [27]. Genomic DNA was extracted using standard methods. PCRs were performed using a final volume of 15 μL for MS-PCR and a final volume of 25 μL for BS-SEQ containing 1× PCR EcoStart buffer (Ecogen), 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 0.25 μM of each primer, and 1.5 U of EcoStart *Taq* polymerase (Ecogen). DNA from normal lymphocytes treated in vitro with *Sss*I methyltransferase and from normal lymphocytes were used as positive controls for methylated and unmethylated alleles, respectively. PCR products were loaded onto nondenaturing 2 % agarose gels, stained with Gel Stain (Lonza, Rockland, USA), and visualized under an UV transilluminator.

Table 1 Distribution of KiSS-1 methylation rates depending on clinical variables

		Training set (n=31)	Validation set (n=100)	Validation set (n=190)
Clinical stage	0	1/2 (50.0)	0/0	0/0
	I	3/7 (42.9)	0/0	6/11 (54.5)
	II	6/10 (60.0)	70/100 (70.0)	68/90 (75.5)
	III	5/9 (55.5)	0/0	78/89 (80.9)
	IV	2/3 (66.7)	0/0	0/0
Histological stage	TIS	1/2 (50.0)	0/0	0/0
	T1	1/2 (50.0)	0/0	0/0
	T2	3/7 (42.9)	15/23 (65.2)	13/16 (81.2)
	T3	8/16 (50.0)	51/69 (73.9)	118/150 (78.6)
	T4	3/5 (60.0)	4/8 (50.0)	21/24 (87.5)
Grade	I	0 %	50/64 (78.1)	13/17 (76.5)
	II	13/26 (50.0)	12/26 (46.2)	112/135 (82.9)
	III	4/6 (66.7)	8/10 (80.0)	27/38 (71.0)
Lymph nodal status	NX	0/0	3/3 (100.0)	0/0
	N0	10/19 (52.6)	67/97 (69.1)	79/101 (78.2)
	N1	3/6 (50.0)	0/0	53/59 (89.8)
	N2	2/4 (50.0)	0/0	20/30 (66.7)
	N3	2/2 (100.0)	0/0	0/0
Metastasis status	MX	4/8 (50.0)	0/0	0/0
	M0	11/20 (55.0)	70/100 (70.0)	152/190 (80.0)
	M+ (M2)	2/3 (66.7)	0/0	0/0

M2 two metastatic sites

RNA and protein analysis of KiSS-1 in CRC cell lines Human CRC cell lines (n=5) were treated with 1 and 5 μmol/L azacitidine (AZA, Sigma) for 72 h to achieve demethylation [27]. RNA was isolated using standard methods. RNA (1 μg) was reverse-transcribed using AMV reverse transcriptase (Promega) and amplified using specific primers and conditions for KiSS-1 [27]. PCR was done using a final volume of 15 μL containing 1× PCR EcoStart buffer (Ecogen), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphate, 0.25 μmol/L of each primer, 1.5 U EcoStart *Taq* polymerase (Ecogen), and 0.4 μg cDNA as template. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control ensuring cDNA quality and loading accuracy. The amplification product was resolved by 2 % agarose gel electrophoresis and visualized by ethidium bromide staining. Cell lysates for protein analysis were analyzed by Western blotting using an anti-KiSS-1 antibody (rabbit polyclonal; Abnova, 1:100 dilution). Equal loading was tested by reprobing with an α-tubulin antibody (mouse monoclonal; Sigma, 1:4,000 dilution).

Tissue microarrays Five tissue microarrays were constructed at the Spanish National Cancer Center and used in this study, including paraffin tissues belonging to patients and controls recruited under IRB-approved protocols at collaborating institutions. From each specimen, triplicate cores with diameters of 1.0 mm were punched and arrayed on the recipient paraffin block. Five-micrometer sections of these tissue array blocks were cut and placed on charged polylysine-coated slides and used for immunohistochemistry analysis. The tissue microarrays included the training set (n=62) and the second validation set (n=190) specimens. Clinicopathologic and annotated follow-up information allowed evaluation of associations of KiSS-1 methylation and protein expression patterns among them and with clinicopathologic variables.

Immunohistochemistry Protein expression patterns of KiSS-1 were assessed at the microanatomic level by immunohistochemistry on these tissue microarrays following standard avidin–biotin immunoperoxidase procedures. Antigen retrieval methods (0.01 % citric acid for 15 min under microwave treatment) were utilized prior to incubation overnight at 4 °C with the primary antibody mentioned above at 1:100 dilution. The biotinylated secondary antibody (Vector Laboratories) was used at 1:1,000 dilution. The absence of primary antibody was used as negative control, while normal urothelium was used as positive control. Diaminobenzidine was utilized as the final chromogen and hematoxylin as the nuclear counterstain.

Serum tumor markers Serum was extracted preoperatively to evaluate the diagnostic and prognostic value of CEA by electrochemiluminescence immunoassay using the

cobas® 6000 autoanalyzer (Elecsys cobas e601, Roche Diagnostics).

Statistical analysis The consensus value of the scores of two independent observers of the three representative cores from each tumor sample arrayed was used for statistical analyses. Associations among KiSS-1 methylation and protein expression patterns of KiSS-1 with clinicopathological variables were evaluated using nonparametric Wilcoxon–Mann–Whitney and Kruskal–Wallis tests [29]. KiSS-1 expression was evaluated as a continuous variable based on the number of cells expressing the protein in the cytoplasm. The intensity of the staining was categorized from negative (–) to low (+), intermediate (++), and high (+++). The diagnostic performance of KiSS-1 methylation measured by MS-PCR in tissue specimens was defined considering the number of correctly classified cases and controls. It was based on the area observed under the curve using receiver operating curve (ROC) analyses, including 95 % confidence interval and statistical significance [29]. Associations of methylation and protein expression patterns with clinical outcome were evaluated in those cases for which follow-up information was available using the log-rank test [29]. The cutoff of protein expression for prognostic evaluation

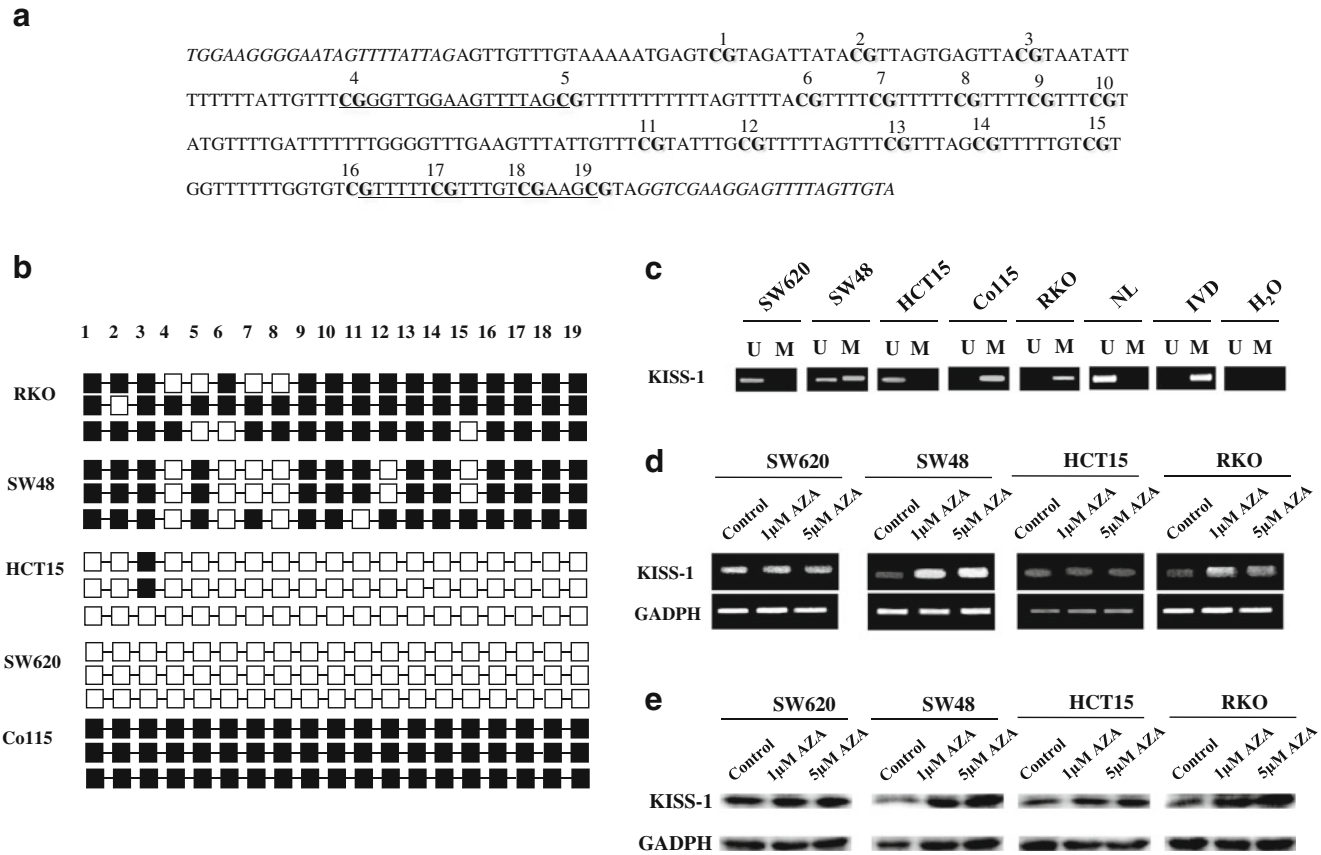

Fig. 1 Identification of KiSS-1 methylation in associated with KiSS-1 silencing. **a** Schematic depiction of the KiSS-1 CpG islands around its transcription start site. Nucleotide sequences of the CpG islands' (in *bold*) region analyzed by bisulfite sequencing, highlighting in *italics* the sequencing primers and *underlining* the primers utilized for MS-PCR. **b** Analysis of CpG islands' methylation status of the promoter of KiSS-1 by bisulfite genomic sequencing in colon human cancer cell lines. Three individual clones are shown per cell line. CpG dinucleotides were represented in *squares*. The presence of "Cs" in the dinucleotide CpG reflects methylated cytosines (*black squares*), while the presence of "Ts" in the dinucleotide CpG reflects unmethylated cytosines (*white squares*). The presence of methylation was confirmed in at least two of the three clones that were sequenced for the cell lines under analyses. **c** Methylation-specific PCRs for KiSS-1 in human colon cancer cell lines. The presence of a PCR band under *lane M* indicates a methylated KiSS-1, while the presence of a PCR band under *lane U* indicates an unmethylated gene. *NL* and in vitro methylated DNA (*IVD*) were used as negative and positive controls for unmethylated and methylated KiSS-1, respectively. **d** The treatment with the demethylating agent AZA reactivated the transcript expression of KiSS-1. Reverse transcription polymerase chain reaction (RT-PCR) analysis of KiSS-1 expression. GAPDH expression is shown as transcript loading control. KiSS-1 transcript expression increased in the methylated cell lines SW48 and RKO after AZA exposure. The unmethylated cell lines (HCT15 and SW620) did not show changes in KiSS-1 expression. **e** The treatment with the demethylating agent AZA reactivated the protein expression of KiSS-1. Immunoblotting of KiSS-1 protein expression in colon cancer cells after AZA exposure. Tubulin expression is shown as loading control. KiSS-1 protein expression increased in the methylated cell lines SW48 and RKO after AZA exposure. The unmethylated cell lines (HCT15 and SW620) did not show changes in KiSS-1 expression

was selected based on the median values of expression among the groups under analyses. Four clinical endpoints were considered: recurrence, progression into metastatic disease, disease-specific, and overall survival. Disease-specific and overall survival time was defined as the months elapsed between surgery and death as a result of disease and death, respectively (or the last follow-up date). Patients who were alive at the last follow-up or lost to follow-up were censored. Survival curves were plotted using the standard Kaplan–Meier methodology [29]. Statistical analyses were performed using the SPSS statistical package (version 17.0).

Results

KiSS-1 is frequently methylated in CRC cells and epigenetically silenced in vitro KiSS-1 was initially tested to be hypermethylated in CRC cells by means of bisulfite genomic sequencing and MS-PCR targeted to the areas surrounding its transcription start site (Fig. 1a). Bisulfite sequencing revealed methylation for three (60.0 %) of the five CRC cell lines analyzed (Fig. 1b). Among the normal tissues analyzed, normal intestine and normal lymphocytes (NL) were found unmethylated at the KiSS-1 CpG island promoter. KiSS-1 methylation patterns observed by sequencing highly confirmed the methylation found by MS-PCR (Fig. 1c). A further link between hypermethylation and gene silencing was established by the treatment of methylated and unmethylated CRC cell lines with a DNA demethylating drug. Exposure of the methylated cell lines to 5-AZA-2′-deoxycytidine (AZA) increased the expression of KiSS-1 at the transcript level (Fig. 1d). Immunoblotting analyses further confirmed that KiSS-1 protein expression increased after AZA exposure (Fig. 1e). HCT15 and SW620 were used as control cell lines to assess the specificity of AZA exposure not to modify KiSS-1 expression in unmethylated CRC cells. Overall, AZA reactivation analyses indicated a high correlation of increasing transcript and protein expression estimates and KiSS-1 methylation status.

KiSS-1 is frequently hypermethylated in primary colorectal tumors: hypermethylation segregated CRC patients from controls Once the functional consequences of KiSS-1 hypermethylation were determined in vitro, we evaluated its impact in human tissue material by MS-PCR (Fig. 2a). KiSS-1 hypermethylation was tested as a CRC-specific event by comparing methylation of ten colorectal tumors and their respective pairs of normal intestine. KiSS-1 methylation was found in 90.0 % of the colorectal tumors and in 10 % of the matching

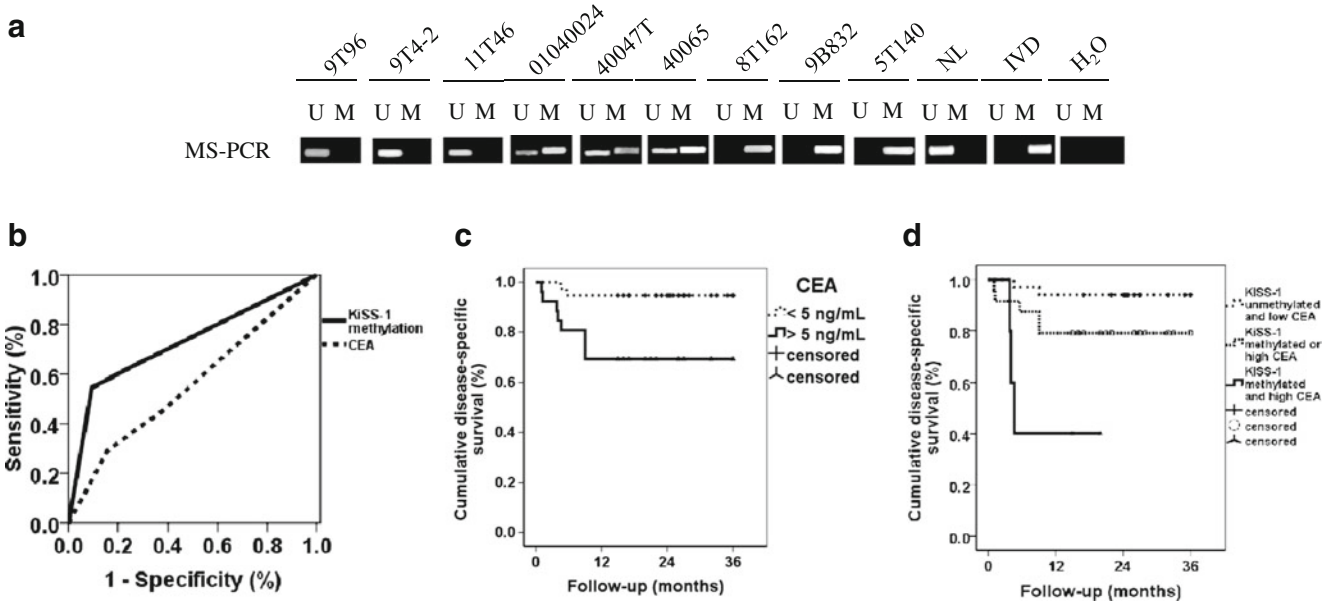

Fig. 2 KiSS-1 hypermethylation plays a role as a diagnostic and prognostic biomarker in colorectal cancer. **a** Representative MS-PCR analyses for KiSS-1 using primary colorectal tumors. Normal lymphocytes (*NL*) and in vitro methylated DNA (*IVD*) were used as negative and positive controls for unmethylated and methylated KiSS-1, respectively. **b** Training set: diagnostic role. Receiver operating curve of KiSS-1 methylation as a detection biomarker for colorectal cancer on a series of 61 tissue specimens collected during colonoscopy or intestinal open surgery. Among these, 31 had colorectal tumors. The area under the curve obtained was 0.727 (95 % confidence interval, 0.599–0.856; $p=0.002$). **c** Training set: CEA prognostic role. Kaplan–Meier curve survival analysis indicating that tumors with higher than 5 ng/mL had poorer disease-specific survival than those with low CEA levels (log rank, $p=0.022$). **d** Training set: Combined prognostic role of CEA and KiSS-1 methylation. Kaplan–Meier curve survival analysis indicating that tumors with higher than 5 ng/mL and methylated for KiSS-1 had poorer disease-specific survival than the rest of combinations and those with low CEA levels and unmethylated for KiSS-1 (log rank, $p=0.001$)

normal intestine analyzed by MS-PCR (data not shown). Initial methylation screening was performed in DNA from paraffin-embedded material belonging to the training set of benign intestine diseases (n=31) and primary colorectal tumors (n=31). ROC analyses provided a global diagnostic accuracy of 72.7 %, as shown by the area under the curve (AUC) (Fig. 2b, AUC=0.727, 95 % confidence interval (CI95 %)=0.599–0.856, p=0.002). Importantly, KiSS-1 methylation showed a higher diagnostic accuracy than preoperative serum CEA levels (Fig. 2b, AUC=0.563, CI95 %=0.416–0.711, p=0.404). These findings supported a diagnostic role for the methylation of KiSS-1 to discriminate between CRC patients and controls with higher diagnostic accuracy than CEA.

KiSS-1 methylation correlated with poor clinical outcome The methylation status of KiSS-1 was then linked to clinicopathologic variables (Table 1). In the pilot training set (n=31), patients with methylation for KiSS-1 showed poorer disease-specific survival, but this trend did not reach statistically significant associations. The prognostic utility of serum CEA alone (Fig. 2c, log rank, p=0.022) was increased when combining with KiSS-1 methylation (Fig. 2d, log rank, p=0.001). These findings prompted us to perform further MS-PCR analyses on two independent validation cohorts of colorectal tumors with available follow-up. In the first validation cohort containing clinical stage II tumors, from which genomic DNA was extracted from frozen material (n=100), KiSS-1 methylation was also a frequent event (70.0 %). Importantly, KiSS-1 methylation

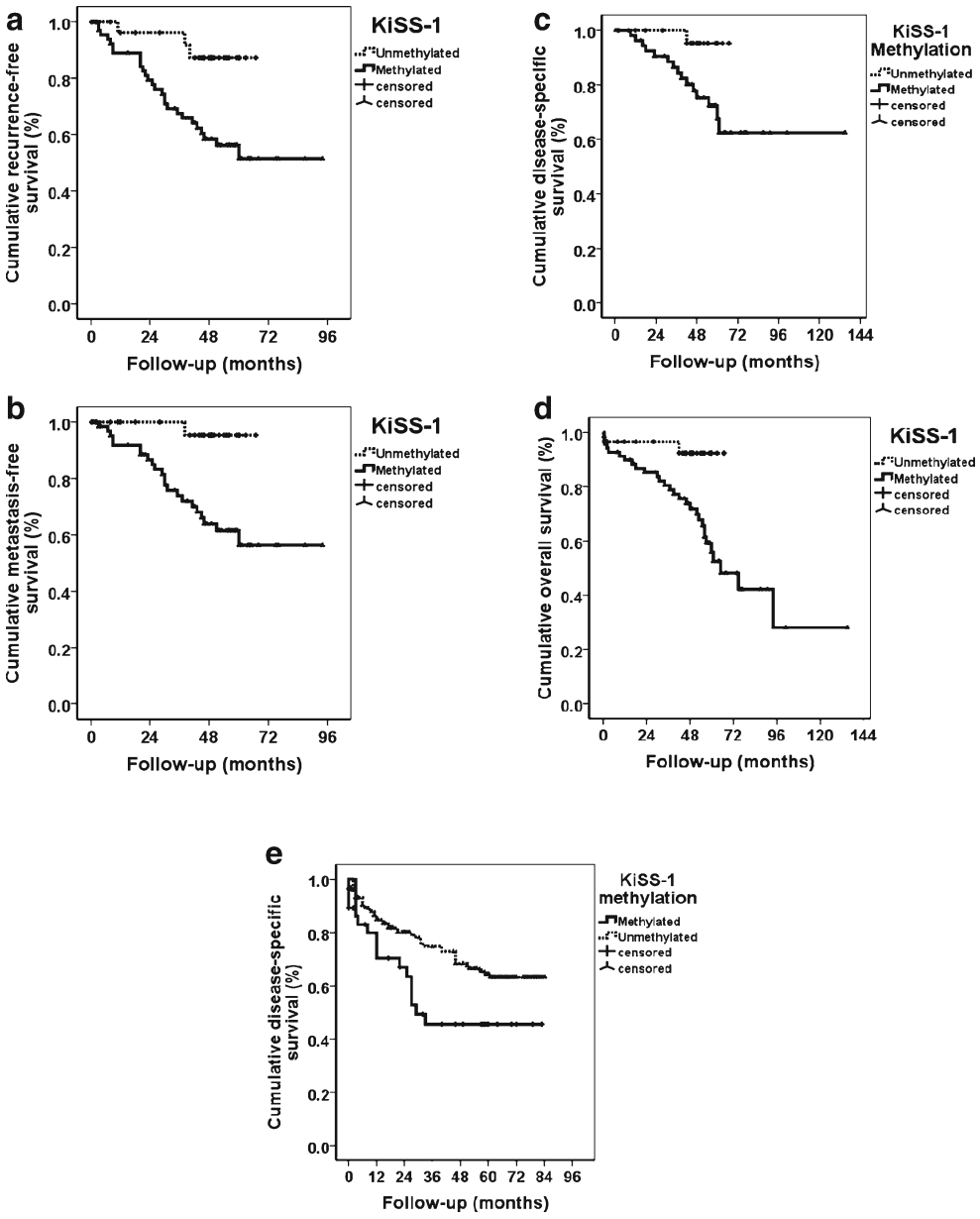

Fig. 3 **a** First validation set: recurrence prognosis. Kaplan–Meier curve survival analysis indicating that tumors with a methylated KiSS-1 had higher recurrence rates than those with unmethylated KiSS-1 (log rank, p=0.009). **b** First validation set: metastasis prognosis. Kaplan–Meier curve survival analysis indicating that tumors with a methylated KiSS-1 had higher metastasis rate than those with unmethylated KiSS-1 (log rank, p=0.004). **c** First validation set: disease-specific survival prognosis. Kaplan–Meier curve survival analysis indicating that tumors with a methylated KiSS-1 had poorer disease-specific survival than those with unmethylated KiSS-1 (log rank, p=0.034). **d** First validation set: overall survival prognosis. Kaplan–Meier curve survival analysis indicating that tumors with a methylated KiSS-1 had shorter overall survival than those with unmethylated KiSS-1 (log rank, p=0.015). **e** Second validation set: disease-specific survival prognosis. Kaplan–Meier curve survival analysis indicating that tumors with a methylated KiSS-1 had poorer disease-specific survival than those with unmethylated KiSS-1 (log rank, p=0.030)

significantly correlated with tumor grade (p=0.011), predicted recurrence (Fig. 3a, log rank, p=0.009), metastasis (Fig. 3b, log rank, p=0.004), disease-free (Fig. 3c, log rank, p=0.034), and overall survival (Fig. 3d, log rank, p=0.015). In the second validation set of colorectal tumors (n=190), KiSS-1 methylation rate was 80.0 %, correlated with lymph node metastasis (Kruskall–Wallis, p=0.012) and predicted poor disease-specific survival (log rank, p=0.030, Fig. 3e). Overall, these results indicated that KiSS-1 hypermethylation was a frequent event in CRC, correlated with clinicopathologic variables and provided prognostic utility for CRC patients.

Loss of cytoplasmic KiSS-1 protein expression patterns correlated with methylation, tumor progression, and clinical outcome in CRC patients The next set of analyses dealt with the evaluation of the protein expression patterns of KiSS-1 in colorectal tissues by immunohistochemistry in the tissue arrays constructed for this study. Protein expression of KiSS-1 by immunohistochemistry was mainly observed in the cytoplasm. In the array containing the training set with those patients with benign intestine diseases and colorectal tumors (n=62), high cytoplasmic KiSS-1 protein expression was found in normal intestine, patients with benign intestine disease, and in early stage adenocarcinomas as compared with advanced colorectal tumors (Fig. 4a, b) (Mann–Whitney, $p \leq 0.0005$). Tumors methylated for KiSS-1 had lower cytoplasmic protein expression than unmethylated cases (Mann–Whitney, p=0.005). In the arrays containing the second validation set (n=190), low cytoplasmic protein expression also correlated with KiSS-1 methylation (p=0.038), tumor grade (p=0.035), and tumor stage (p=0.007). Tumors with low cytoplasmic expression were more prone to recurrence (log rank, p=0.017; Fig. 4c) and showed shorter disease-specific survival (log rank, p=0.020; Fig. 4d). Overall, these analyses in two independent cohorts revealed that the loss of KiSS-1 cytoplasmic protein correlated with methylation, tumor staging, and poor clinical outcome for CRC patients. Thus, KiSS-1 methylation and its cytoplasmic expression could be considered likely prognosticators of tumor progression and poor outcome in CRC patients.

Discussion

This retrospective proof of principle study identified the differential expression and the epigenetic silencing of KiSS-1 in CRC. Importantly, KiSS-1 methylation and its expression were clinically relevant for the diagnostic and prognostic assessment of CRC patients. The consequences of KiSS-1 hypermethylation along CRC progression require to be assessed from the standpoint of mechanistic and translational implications. Mechanistically, it is important to link the promoter methylation status to expression estimates of KiSS-1. AZA exposure experiments confirmed the effect of methylation on KiSS-1 expression by restoring KiSS-1 transcript and protein expression specifically in methylated CRC cells. In concordance, KiSS-1 cytoplasmic expression was significantly lower in methylated than in unmethylated colorectal tumors. Thus, methylation correlated with loss of KiSS-1 expression also in human clinical material. Overall,

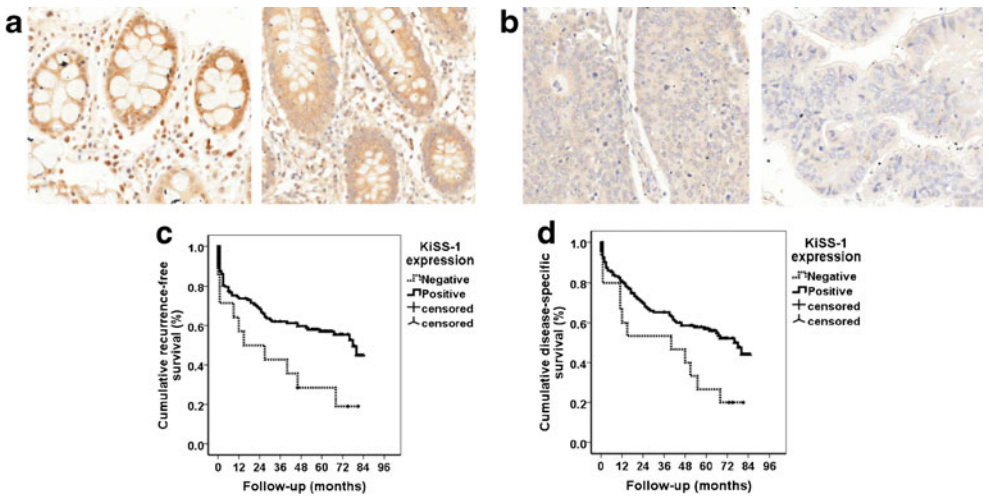

Fig. 4 Protein expression patterns of KiSS-1 correlated with tumor progression and clinical outcome in colorectal cancer. **a**, **b** Representative immunostainings of the differential protein expression patterns of KiSS-1 at the tissue level by immunohistochemistry on tissue arrays in **a** normal intestinal epithelium and proliferative adenomatous polyps and **b** colorectal tumors. **c** Validation set: recurrence. Kaplan–Meier curve survival analysis indicating that negative cytoplasmic protein expression of KiSS-1 correlated with high recurrence rates (log rank, p=0.017). **d** Validation set: disease-specific survival. Kaplan–Meier curve survival analysis indicating that patients with negative cytoplasmic protein expression of KiSS-1 correlated with poor disease-specific survival (log rank, p=0.022)

in vitro and in vivo tissue human analyses revealed that KiSS-1 was aberrantly silenced by CpG island promoter hypermethylation.

To our knowledge, the expression of KiSS-1 has not been described in CRC. In bladder tumors, the loss of KiSS-1 expression correlated with increased tumor staging and poor clinical outcome [16] and was later attributed to epigenetic silencing by hypermethylation [27]. The epigenetic silencing identified in CRC is consistent with the mechanism by which KiSS-1 is lost in bladder progression [27]. In this report, the impact and clinical relevance of KiSS-1 methylation along CRC development was evaluated using in vitro strategies and on tissue specimens. Biologically, *KISS1* was reported to play a metastasis suppressor role in neoplastic diseases. Indeed, KiSS-1 methylation was shown to correlate with the presence of lymph node metastases in CRC patients in one of the validation sets. Our results revealed that KiSS-1 was hypermethylated and potentially useful as a diagnostic and tumor stratification biomarker and clinical outcome prognosticator for CRC patients.

The translational implications of the discovery of KiSS-1 methylation in CRC have strongly been addressed in this work. The association of KiSS-1 with CRC progression can be justified as follows: First, the cancer specificity evaluation by MS-PCR in pairs of colorectal tumors and matching normal intestinal epithelium suggested that KiSS-1 methylation was a cancer event, supported by the significant expression differences at the protein level. Second, KiSS-1 methylation significantly discriminated CRC from benign intestinal disease. Third, KiSS-1 methylation was a frequent event in three independent series of tumors with over 350 tissues analyzed, regardless of the source of genomic DNA from frozen or paraffin-embedded tissue material. Fourth, it correlated with tumor staging, including lymph node metastasis. Furthermore, it added prognostic information of clinical outcome. In addition to the clinicopathologic stratification of CRC patients, a relevant translational point relates to treatment. In this new scenario, KiSS-1 represents a potential therapeutic target whose expression could be potentially reactivated by demethylating drugs and also by means of kisspeptins [18, 25].

A critical step in the clinical evaluation of KiSS-1 along CRC progression deals with analyses of KiSS-1 protein expression patterns by immunohistochemistry on colorectal tumors of known KiSS-1 methylation status. The loss of KiSS-1 cytoplasmic protein expression correlated with increasing methylation rates, tumor stage, and tumor grade. Interestingly, low cytoplasmic KiSS-1 protein levels were also correlated with poor clinical outcome under different clinical endpoints, suggesting its potential role as a prognostic marker for the clinical management of CRC patients. Thus, epigenetic and protein analyses revealed that KiSS-1 is differentially expressed along CRC progression in association with clinicopathologic variables and clinical outcome. The epigenetic silencing of KiSS-1 might aid understanding as to how it may contribute to tumor progression in CRC. Future studies are warranted to dissect such specific mechanisms in the context of colorectal tumors and other solid human neoplasias.

The clinical diagnostic utility of KiSS-1 methylation patterns in tissue specimens represents a relevant finding. KiSS-1 methylation discriminated CRC patients from benign intestinal disease with higher diagnostic accuracy than preoperative CEA serum levels. An additional goal was to assess how methylation could further discriminate clinical outcome in combination with CEA. Importantly, KiSS-1 methylation also improved the prognostic utility of CEA, a serum biomarker frequently used in clinical practice to monitor patients with CRC [30]. Thus, assessing KiSS-1 methylation represents a potential alternative adjunct for the early detection of patients under suspicion of CRC using biopsy specimens and potentially for the follow-up of CRC patients.

In summary, our study revealed that KiSS-1 is differentially expressed in CRC. It provided a mechanistic explanation for the identified loss of KiSS-1 cytoplasmic expression in colorectal malignancies by epigenetic silencing. Hypermethylation emerged as a strong indicator of tumor aggressiveness for CRC patients. The loss of cytoplasmic KiSS-1 protein expression also stratified colorectal tumors histopathologically and predicted clinical outcome. Interestingly, KiSS-1 methylation in tissue specimens played a higher diagnostic and complementary prognostic accuracy than CEA for CRC. These observations support introducing KiSS-1 assessment for the stratification and clinical management of CRC patients.

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Conflicts of interest None

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