

Short Communication

KISS1 Methylation and Expression as Tumor Stratification Biomarkers and Clinical Outcome Prognosticators for Bladder Cancer Patients

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KISS1 is a metastasis suppressor gene that is lost in several malignancies, including bladder cancer. We tested the epigenetic silencing hypothesis and evaluated the biological influence of KISS1 methylation on its expression and clinical relevance in bladder cancer. KISS1 hypermethylation was frequent in bladder cancer cells analyzed by methylation-specific PCR and bisulfite sequencing and was associated with low gene expression, being restored *in vitro* by demethylating azacytidine. Hypermethylation was also frequently observed in a large series of bladder tumors (83.1%, $n = 804$). KISS1 methylation was associated with increasing stage ($P = 0.001$) and tumor grade ($P = 0.010$). KISS1 methylation was associated with low KISS1 transcript expression by quantitative RT-PCR ($P = 0.037$). KISS1 transcript expression was also associated with histopathological tumor stage ($P < 0.0005$). Low transcript expression alone ($P = 0.003$) or combined with methylation ($P = 0.019$) was associated with poor disease-specific survival ($n = 205$).

KISS1 transcript expression remained an independent prognosticator in multivariate analyses ($P = 0.017$). KISS1 hypermethylation was identified in bladder cancer, providing a potential mechanistic explanation (epigenetic silencing) for the observed loss of KISS1 in uroepithelial malignancies. Associations of KISS1 methylation and its expression with histopathological variables and poor survival suggest the utility of incorporating KISS1 measurement using paraffin-embedded material for tumor stratification and clinical outcome prognosis of patients with uroepithelial neoplasias. (Am J Pathol 2011, 179:540–546; DOI: 10.1016/j.ajpath.2011.05.009)

Bladder cancer can be described as a molecular disease, driven by the multistep accumulation of genetic and epigenetic factors.¹ The most common epigenetic event is the addition of methyl groups to the carbon-5 position of cytosine nucleotides.² CpG islands are present in one-half of human genes.^{3–6} CpG island hypermethylation has been associated with the transcriptional inactivation of cancer-related genes, including in bladder cancer.^{6–8}

KISS1 was identified as suppressing metastases in melanoma and breast cancer cells.^{9–11} The KISS1 gene maps to chromosome region 1q32,¹² and it can be regulated by genes located on chromosome 6.^{10,11,13} The highest concentrations of the KISS1 protein are found in the placenta, although it is also expressed in the central nervous system, testis, ovary, pancreas, and intestine.^{9–11} KISS1 encodes a 145-amino acid protein that is

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Table 1. Primer Sequences and PCR Conditions for *KISS1* Analyses

Analysis	Sense primer	Antisense primer	Product size (bp)	T _{anneal} (°C)	PCR cycles (no.)
BS-SEQ	5'-TGGAAGGGGAATAGTTTTATTAG-3'	5'-TACAACATAAACTCCTTCRACC-3'	322	52	40
MSP	5'-CGGGTTGGAAGTTTTAGC-3'	5'-GCTTCGACAAACGAAAAAC-3'	125	54	37
USP	5'-TTTTGGGTTGGAAGTTTTAGT-3'	5'-ACTTCAACAAACAAAAACAAC-3'	129	52	37
RT-PCR	5'-ACTCACTGGTTTCTTGGCAGC-3'	5'-ACCTTTTCTAATGGCTCCCA-3'	183	60	28

BS-SEQ, bisulfite sequencing; MSP, methylation-specific PCR; T_{anneal}, annealing temperature; USP, unmethylated specific PCR.

ated, cytosine to uracil. First, genomic sequencing of both DNA strands of *KISS1* was analyzed after bisulfite treatment (BS-SEQ), for at least three clones per bladder cancer cell line, as previously reported.^{8,29} Confirmation on at least two independent clones was required to assign methylation sites (Figure 1B). A second strategy used PCR with primers (Figure 1A and Table 1) specific for either the methylated or the modified unmethylated DNA (MS-PCR).^{8,29} PCRs were performed using a final volume of 15 μ L for MS-PCR and a final volume of 25 μ L for BS-SEQ containing 1 \times PCR EcoStart buffer (Ecogen, Barcelona and Madrid, Spain), 1.5 mmol/L of MgCl₂, 0.2 mmol/L of dNTP, 0.25 μ mol/L of each primer and 1.5 U of EcoStart Taq polymerase (Ecogen). Genomic DNA was extracted using standard methods. DNA from normal lymphocytes treated *in vitro* (IVD) with SssI methyltransferase was used as a positive control for methylated alleles. DNA from normal lymphocytes and B-cell chronic lymphocytic leukemia cells (MEC1) was used as a positive control for unmethylated alleles. PCR products were loaded onto nondenaturing 2% agarose gels, stained with ethidium bromide, and visualized under an UV transilluminator.^{8,29}

RNA Analysis of *KISS1* in Bladder Cancer Cell Lines

Human bladder cancer cell lines ($n = 11$) were treated with 1 μ mol/L and 5 μ mol/L azacytidine (AZA) (Sigma-Aldrich, St. Louis, MO) for 72 hours to achieve demethylation.^{8,29} RNA was isolated using standard methods. RNA (1 μ g) was reverse-transcribed using AMV reverse transcriptase (Promega, Madison, WI) and amplified using specific primers and conditions for *KISS1* (Table 1). PCR was performed using a final volume of 15 μ L containing 1 \times PCR EcoStart buffer (Ecogen), 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTP, 0.25 μ mol/L of each primer, and 1.5 U EcoStart Taq polymerase (Ecogen). For PCR amplification, 0.4 μ g cDNA was used. *GADPH* was used as internal control to ensure cDNA quality and loading accuracy. The amplification product was resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.^{8,29}

Transcript Expression Analysis by Real-Time Quantitative PCR in Bladder Tumors

KISS1 transcript expression was assessed in paraffin-embedded tumors among patients evaluated for *KISS1* methylation and with available follow-up data (as de-

scribed above). RNA was extracted using the TRIzol (Invitrogen, Carlsbad, CA) method. Complementary DNA was synthesized using a ThermoScript RT-PCR system (Invitrogen). Template cDNA was added to TaqMan universal master mix (Applied Biosystems, Foster City, CA) in a 15- μ L reaction with specific primers and probes acquired from Applied Biosystems for *KISS1* (Hs_00158486_m1), and the chaperonin containing T-complex protein 1 subunit 6A (zeta 1) (*CCT6A*) (Hs_00798979_s1). Quantification of gene expression was performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems). Relative gene expression quantification was calculated according to the comparative cycle threshold (C_T) method using *CCT6A* as endogenous control. Final results were estimated as $2^{-(\Delta C_T \text{ KISS1})}$, where the ΔC_T *KISS1* values for each sample are determined by subtracting the C_T value of the target *KISS1* gene from the value of the *CCT6A* control gene. Only triplicates with standard deviations of the C_T value of <0.20 were accepted.

Statistical Analysis

Associations among *KISS1* methylation and its transcript expression with tumor stage and grade were evaluated using nonparametric Wilcoxon-Mann-Whitney and Kruskal-Wallis tests.³⁰ *KISS1* transcript cutoff was selected based on the median values of expression among the groups under analysis. Combinations of the presence or absence of methylation and *KISS1* transcript levels higher or lower than the selected cutoff were submitted to clinical outcome assessment. Associations of methylation and *KISS1* transcript expression with disease-specific survival were evaluated in those cases with available follow-up data, using the log-rank test.³⁰ Disease-specific survival time was defined as the years elapsed between surgery and death from disease (or the date of the last follow-up visit). Patients who were alive at the last follow-up date or lost were censored. Survival curves were plotted using Kaplan-Meier methodology. To test whether *KISS1* methylation and transcript expression were independent risk factors, a multivariate analysis was performed using a Cox model including also variables such as age, sex, tumor stage, and tumor grade. All analyses were performed with the SPSS software package version 17.0 (SPSS, Chicago, IL).

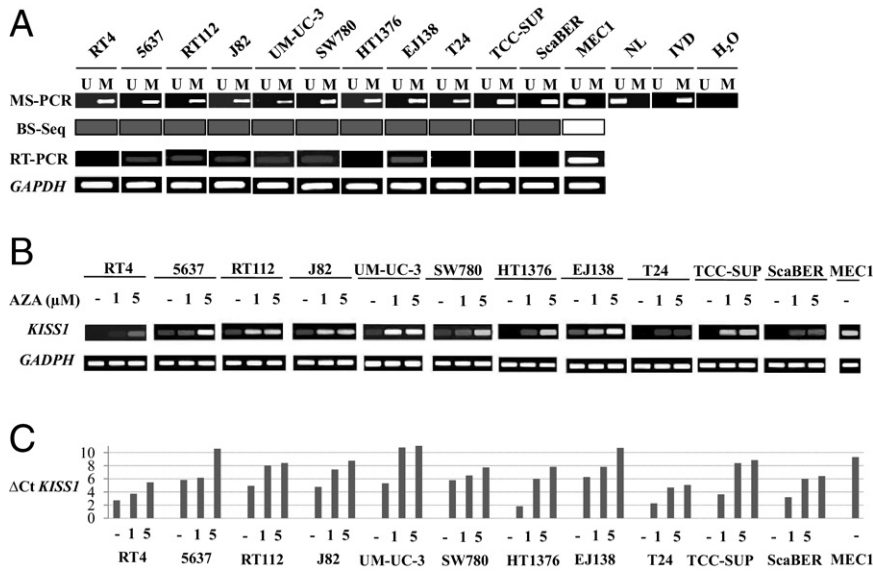


Figure 2. CpG island methylation was associated with *KISS1* silencing. **A:** Methylation-specific PCRs (MS-PCR) for *KISS1* in human bladder cancer-cell lines. A PCR band in lane M indicates a methylated *KISS1* gene; in lane U, it indicates an unmethylated gene. Normal lymphocytes (NL) and MEC1 were used as controls for unmethylated *KISS1*; *in vitro* methylated DNA (IVD) was used as control for methylated *KISS1*. From bisulfite genomic sequencing (BS-Seq), methylated cell lines are highlighted in dark color. With RT-PCR, *GAPDH* expression was used as transcript loading control. **B:** Treatment with the demethylating agent reactivated the transcript expression of *KISS1* in methylated bladder cancer cell lines. Qualitative RT-PCR analysis of *KISS1* expression, with *GAPDH* as transcript loading control. After 1 or 5 μmol/L AZA exposure, *KISS1* transcript expression increased in all of the methylated bladder cancer cell lines. **C:** Quantitative RT-PCR analysis of *KISS1* expression. ΔC_T *KISS1* values for each sample were determined by subtracting the C_T value of the target *KISS1* gene from the value of the *CCT6A* control gene. *KISS1* transcript expression increased in all of the methylated bladder cancer cell lines, after 1 or 5 μmol/L AZA exposure. MEC1, an unmethylated cell line, was used as a positive control for *KISS1* expression.

Results

KISS1 Is Epigenetically Silenced In Vitro

Eleven human bladder cancer cell lines were initially screened using bisulfite genomic sequencing and MS-PCR (Figure 1A). The BS-SEQ revealed CpG island methylation for all of the bladder cancer cell lines analyzed (Figure 1B), in agreement with the MS-PCR results (Figure 2A). Methylation analyses were then linked with *KISS1* expression estimates by RT-PCR. The hypermethylated cell lines showed low transcript expression (Figure 2A). A further link between hypermethylation and gene silencing was established by treatment of bladder cancer cell lines with a demethylating agent. Exposure of the methylated cells to AZA increased the transcript expression of *KISS1* both by qualitative (Figure 2B) and by quantitative RT-PCR analyses (Figure 2C). The degree of demethylation after azacytidine exposure of bladder cancer cells was assessed by BS-SEQ (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Overall, these results indicated a high correlation of *KISS1* methylation with loss of transcript expression *in vitro*, observations especially supported by AZA reactivation analyses.

KISS1 Is Frequently Hypermethylated in Primary Bladder Tumors and Is Associated with Tumor Progression

Once the effect of *KISS1* CpG island hypermethylation on expression was determined *in vitro*, we evaluated its clinical impact in human bladder tumors. The methylation status of *KISS1* was analyzed by MS-PCR in a set of frozen bladder tumors ($n = 25$), for which their respective paired normal urothelium was available (Figure 3A). Comparison of methylation of these pairs of 25 bladder tumors and normal urothelium revealed that *KISS1* methylation was a frequent cancer-specific epigenetic event (Figure 3B). Importantly, *KISS1* was found to be unmethylated in normal urothelium obtained from 10 cystopro-

tatectomized patients with prostate cancer (Figure 3C), supporting the bladder cancer specificity of *KISS1* methylation. High concordance was found between MS-PCR results obtained from genomic DNA from frozen and matching paraffin-embedded tumor material (100%). These observations prompted us to perform further MS-PCR analyses on independent sets of bladder tumors, extracting genomic DNA from paraffin-embedded material. Of note, a high methylation rate for *KISS1* was observed (83.1%) using an independent large set of bladder tumors ($n = 804$). For validation analyses, the methylation status of a subset of the tumors analyzed by MS-PCR was confirmed by BS-SEQ (see Supplemental Figure S2 at <http://ajp.amjpathol.org>).

The next analyses dealt with evaluating the link between the hypermethylation status of *KISS1* and clinicopathological variables. Tumors displaying advanced disease (stage T2 or higher) were more frequently methylated than those presenting non-muscle-invasive lesions (Table 2). Increased methylation rates were observed in high-grade bladder tumors, compared with low-grade lesions (Table 2). Of note, *KISS1* methylation was significantly associated with increased stage (Kruskal-Wallis, $P = 0.001$) and grade ($P = 0.010$). Overall, these results indicate that *KISS1* hypermethylation is a frequent event in bladder cancer in association with pathological indicators of tumor progression.

KISS1 Transcript Expression Correlates with Methylation, Tumor Progression, and Clinical Outcome of Patients with Bladder Tumors

The next set of analyses dealt with the assessment of transcript expression of *KISS1* by quantitative RT-PCR in bladder tumors for which the *KISS1* methylation status was analyzed. Higher *KISS1* expression was found in low-grade and early lesions, compared with high-grade

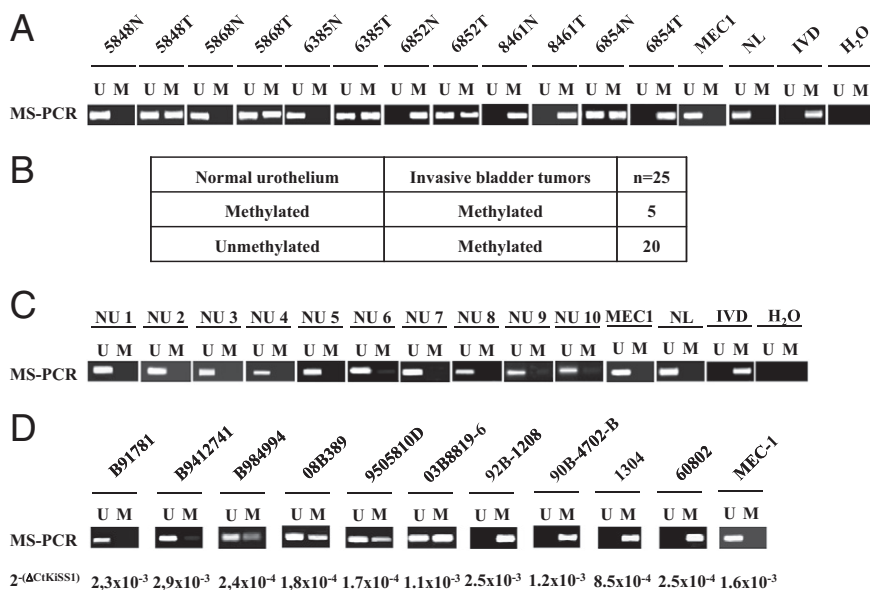


Figure 3. *KISS1* hypermethylation is a frequent bladder cancer-specific event. **A:** *KISS1* hypermethylation was shown to be a cancer-specific event, as indicated by representative MS-PCR analyses for *KISS1* using primary bladder tumors and normal urothelium. Normal lymphocytes (NL) and MEC1 were used as controls for unmethylated *KISS1*; *in vitro* methylated DNA (IVD) was used as control for methylated *KISS1*. **B:** *KISS1* methylation increased in bladder tumors, compared with their paired normal urothelium, as indicated by methylation status of *KISS1* analyzed by MS-PCR in pairs of primary bladder tumors and normal urothelium. **C:** *KISS1* was unmethylated in normal urothelium unrelated to bladder cancer. Representative MS-PCR analyses are shown for *KISS1* in normal urothelium (NU) from cystoprostatectomized patients with prostate cancer. MEC1 cells were used as positive control for unmethylated *KISS1*. **D:** *KISS1* methylation correlated with a low transcript expression. Representative MS-PCR analyses are shown for *KISS1* and matching transcript levels of *KISS1* in bladder tumors.

and invasive bladder tumors. Low transcript levels of *KISS1* were significantly associated with increasing stage (Kruskal-Wallis, $P < 0.0005$) and grade (Mann-Whitney, $P = 0.024$). Tumors methylated for *KISS1* had lower transcript expression than did unmethylated tumors ($P = 0.037$; Figure 3D).

Table 2. Summary of the Distribution of CpG Island Methylation Status of *KISS1* Obtained by Methylation-Specific PCR with Respect to Tumor Stage and Grade in a Series of 804 Bladder Tumors

Variable	No.	%
Stage*		
pTa	69	
Methylated	56	81.2
Unmethylated	13	18.8
pT1	402	
Methylated	318	79.1
Unmethylated	84	20.9
pTis	10	
Methylated	8	80.0
Unmethylated	2	20.0
pT2	187	
Methylated	160	85.6
Unmethylated	27	14.4
pT3	93	
Methylated	86	92.5
Unmethylated	7	7.5
pT4	43	
Methylated	40	93.1
Unmethylated	3	6.9
Grade†		
Low	132	
Methylated	103	78.1
Unmethylated	29	21.9
High	672	
Methylated	565	84.1
Unmethylated	107	15.9

**KISS1* methylation was associated with increasing tumor stage ($P = 0.001$).

†*KISS1* methylation was associated with increasing tumor grade ($P = 0.01$).

In a subset of 205 bladder tumors with available follow-up data, having a transcript expression of *KISS1* with a 2^{-ΔCt} value of <0.1 was significantly associated with shorter disease-specific survival (log rank, $P = 0.003$; Figure 4A). Although *KISS1* methylation alone was not significantly associated with disease-specific survival (log rank, $P = 0.23$; Figure 4B), the combined presence of a *KISS1* transcript expression with a 2^{-ΔCt} value of <0.1 and *KISS1* methylation was strongly associated with the poorest survival (log rank, $P = 0.019$; Figure 4C). Lack of statistically significant associations were found when performing log-rank statistics comparing only those patients with low *KISS1* expression and methylated for *KISS1* versus those with low *KISS1* expression and unmethylated for *KISS1*, as well as only those with high *KISS1* expression and methylated for *KISS1* versus those with high *KISS1* expression and unmethylated for *KISS1* (Figure 4C). These analyses indicated the influence of a high expression of *KISS1* on a favorable outcome. Multivariate Cox analyses indicated that tumor stage and *KISS1* expression were the only independent prognostic factors ($P = 0.001$), having hazard ratios of death of 2.62 (95% CI = 1.49 to 4.58, $P = 0.001$) and 0.42 (95% CI = 0.20 to 0.85; $P = 0.017$), respectively.

Overall, these analyses revealed that the loss of transcript expression was associated with methylation, tumor stage, and poor survival. The combination of *KISS1* methylation and low transcript expression was also related to poor disease-specific survival. Thus, these variables could be considered tumor stratification biomarkers and likely prognosticators of poor outcome for bladder cancer patients.

Discussion

The present study revealed the epigenetic silencing of *KISS1* in bladder cancer and showed the clinical relevance of *KISS1* methylation in uroepithelial tumors. The

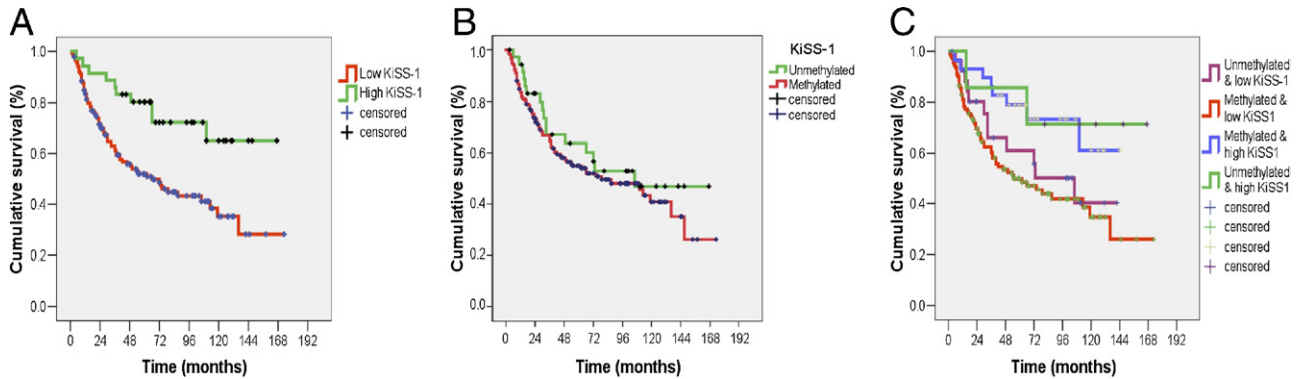


Figure 4. Transcript expression and *KISS1* methylation are associated with clinical outcome in bladder cancer. **A:** Loss of *KISS1* expression alone was associated with disease-specific survival. Kaplan-Meier survival curve analysis indicated that low transcript expression of *KISS1* with a $2^{-\Delta C_t \text{ KISS1}}$ value of < 0.1 measured by RT-PCR was associated with poor survival (log rank, $P = 0.003$). **B:** *KISS1* methylation alone was not significantly associated with disease-specific survival. Kaplan-Meier survival curve analysis indicated that tumors methylated for *KISS1* had poorer survival than unmethylated tumors, but this trend did not reach statistical significance (log rank, $P = 0.238$). **C:** Loss of *KISS1* transcript expression in combination with *KISS1* methylation was associated with disease-specific survival. Kaplan-Meier survival curve analysis indicated that tumors with a transcript expression of *KISS1* with a $2^{-\Delta C_t \text{ KISS1}}$ value of < 0.1 and methylated for *KISS1* had the poorest survival (log rank, $P = 0.019$).

consequences of the novel CpG island hypermethylation of *KISS1* in bladder cancer can be assessed from the standpoint of mechanistic and translational implications. Mechanistically, it is important to evaluate the cellular consequences of *KISS1* methylation. Expression estimates of *KISS1* were linked to methylation analyses using several approaches. Methylation status and transcript expression of *KISS1* correlated to a high extent among a variety of bladder cancer cell lines. AZA exposure experiments confirmed the biological effect of methylation in *KISS1* expression by specifically restoring *KISS1* transcript expression in methylated cells. Overall, *in vitro* analyses revealed that *KISS1* was aberrantly silenced by CpG island hypermethylation. Along this line of argument, a statistically significant association was found between methylation and low transcript expression of *KISS1* by RT-PCR. In bladder tumors, the increased methylation rate together with the loss of transcript expression of *KISS1* was also found to be associated with increasing tumor stage and poor clinical outcome. Thus, increased methylation rates correlated with loss of *KISS1* expression and aggressive bladder cancer also in human clinical material. Biologically, *KISS1* is reported to play a metastasis suppressor role in neoplastic diseases. Although its role in cancer progression has not been elucidated, the epigenetic silencing of *KISS1* might aid understanding as to how *KISS1* may contribute to bladder cancer. Further studies are warranted to dissect the influence of *KISS1* hypermethylation on its expression in the context of other malignancies.

The translational implications of the discovery of *KISS1* methylation have been strongly addressed in the present study. The association of *KISS1* methylation with bladder cancer can be justified as follows. First, the cancer specificity was supported by MS-PCR analyses in pairs of bladder tumors and normal urothelium. It is important to be aware of the potential influence of the selected high number of cycles on detecting hypermethylation in normal urothelium counterparts in a tumor characterized by displaying field effect. Second, it was shown, using a large series of bladder cancer

patients ($n = 804$), that the methylation of *KISS1* is a frequent event among bladder tumors, regardless of the source of genomic DNA obtained from frozen or paraffin-embedded material. Methylation analyses were performed after bisulfite treatment using low amounts of DNA (500 to 1000 ng). Although bisulfite treatment is feasible using lower amount of nucleic acids, DNA quantity could be a potential limitation for low-stage and low-grade small tumors. Third, *KISS1* methylation was significantly associated with increasing tumor stage and tumor grade. In addition to the clinicopathologic stratification of bladder cancer patients, a relevant translational point relates to treatment. In this new scenario, *KISS1* represents a potential therapeutic target whose expression could be potentially reactivated by demethylating drugs, and also by means of administration of kisspeptins.^{18,25}

A further step in the translational implications of the clinical evaluation of *KISS1* in bladder cancer deals with analyses of its transcript expression by RT-PCR on bladder tumors of known *KISS1* methylation status. These analyses served not only to confirm the previously reported clinical relevance of the loss of expression in bladder cancer correlating with increasing tumor stage and grade,^{12,22} but also to link the low transcript expression with the identified potential methylation mechanism of inactivation. An additional goal was to assess how methylation could further discriminate clinical outcome in combination with transcript expression. Notably, the presence of low transcript levels alone or in combination with methylation was associated with poor survival. *KISS1* methylation stratified the discrimination among patients with good or poor outcome obtained by transcript expression alone. The multivariate analysis revealed that *KISS1* transcript expression remained an independent prognosticator. Thus, the combination of methylation and transcript analyses has served to discover the epigenetic silencing of a gene with reported low expression. Moreover, these variables were significantly associated with clinicopathologic correlates and clinical outcome.

Conclusions

KISS1 hypermethylation was identified in bladder cancer, providing a potential mechanistic explanation for the observed loss of *KISS1* in uroepithelial malignancies by epigenetic silencing. *KISS1* expression showed higher correlations with clinical outcome than did methylation, suggesting that DNA methylation correlates with silencing but that it might not be the only mechanism driving silencing. Associations of *KISS1* methylation and its expression with histopathological variables and poor survival suggest the utility of incorporating these *KISS1* measurements using paraffin-embedded material for tumor stratification and for clinical outcome prognosis of patients affected with uroepithelial neoplasias.

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