CALN1 Hypomethylation As A Biomarker For High-Risk Bladder Cancer

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Research Article

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Abstract

Background: DNA methylation in cancer is considered a diagnostic and predictive biomarker. We investigated the usefulness of the methylation status of *CALN1* as a biomarker for bladder cancer using methylation-sensitive restriction enzyme (MSRE)-quantitative polymerase chain reaction (qPCR).

Methods: A total of 82 bladder cancer fresh samples were collected via transurethral resection of bladder tumors. Genomic DNA was extracted from samples, and MSRE-qPCR was performed to determine the *CALN1* methylation percentage. Reverse transcription-qPCR was performed to assess the correlation between *CALN1* methylation and mRNA expression. The association between *CALN1* methylation percentage and clinicopathological variables of all cases and intravesical recurrence of non-muscle-invasive bladder cancer (non-MIBC) cases were analyzed.

Results: Of the 82 cases, nine were MIBC cases and 71 were non-MIBC cases that had not undergone total cystectomy. The median *CALN1* methylation percentage was 79.5% (interquartile range: 51.1, 92.6). *CALN1* gene methylation percentage had a negative relationship with *CALN1* mRNA expression (Spearman's ρ = -0.563 and P = 0.0121). Hypomethylation of *CALN1* was associated with advanced tumor stage (P = 0.0007) and histologically high grade (P = 0.0178). Furthermore, multivariate analysis revealed that *CALN1* hypomethylation was an independent risk factor for intravesical recurrence in non-MIBC patients (hazard ratio 3.83, 95% confidence interval; 1.14–13.0, P = 0.0305).

Conclusion: Our findings suggest that *CALN1* methylation percentage could be a useful molecular biomarker in bladder cancer patients.

Background

Bladder cancer is a common malignancy worldwide. Once muscle-invasive bladder cancer (MIBC) progresses, it is difficult to control, and its prognosis is poor. Moreover, the risk of intravesical recurrence of non-MIBC is high, and the risk of progression to MIBC is not necessarily low [1], even though non-MIBC has a relatively good prognosis.

Cystoscopy is the most effective technique for diagnosing bladder cancer recurrence but is highly invasive. Urine cytopathology is currently widely used for diagnosis, but its sensitivity for detecting bladder cancer is low and reportedly depends on the skill of the cytopathologist [2]. Although other methods, using several biomarkers and nucleic acid probes such as bladder tumor antigen [3], nuclear matrix protein 22 [4], and UroVysion™ fluorescence in situ hybridization [5], have been developed, the robustness of these methods for early detection of bladder cancer and risk stratification in clinical practice has not been established. Therefore, there is an urgent need to establish new biomarkers.

In recent years, DNA methylation, as an epigenetic mechanism that regulates gene expression without changing the base sequence, has attracted great attention, and DNA methylation status in bladder cancer

has been widely studied [6]. Inactivation of gene expression due to promoter methylation can be a useful biomarker for diagnosis and prognosis [7–9].

We previously conducted a preliminary experiment focused on *CALN1*, using the Ion Ampliseq™ Methylation Panel for Cancer Research, and found that *CALN1* is associated with the clinicopathological features of bladder cancer (unpublished data). *CALN1* encodes a protein that is highly similar to the calcium-binding proteins of the calmodulin family [10]. Calcium signaling is an important regulator in various cellular processes and has been implicated in important activities related to cancer progression, such as proliferation and infiltration [11, 12]. In this study, we investigated the usefulness of determining *CALN1* methylation status as a biomarker for bladder cancer.

Methods

Study population

Eighty-two patients who underwent transurethral resection of bladder tumor (TURBT) between April 2019 and June 2021 at the Diyukai Daiichi Hospital were enrolled in this study. Data on age, sex, smoking status, and tumor stage, grade, number, size, and type (primary/recurrent) were collected. The study was performed following approval from the Ethics Committee of the Shakai Iryo Hojin Daiyukai (approval no.2019002) and was conducted in accordance with the Declaration of Helsinki.

Genomic (g)DNA isolation

The collected tissues were washed with saline and stored immediately at -80 ℃. Genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Molecular Systems, Pleasanton, CA, USA) according to the instruction manual, and the eluate (100 µL of elution buffer) was used for further analysis.

Restriction enzyme treatment

Isolated DNA (100 ng gDNA) was treated with Hap II (Takara Bio, Shiga, Japan), a methylation-sensitive restriction enzyme, and/or Msp I (Takara Bio), a methylation-independent restriction enzyme, according to the manufacturer's instruction. Hap II and Msp I are isoschizomers of each other. Hap II does not cleave the methylated recognition sequence while Msp □ cleaves regardless of methylation status.

Quantitative polymerase chain reaction (qPCR)

Following enzymatic treatment, a quantitative DNA methylation analysis was performed using qPCR. Primers were designed using the intron 2 sequence of *CALN1* with the GenBank accession number NC_000007.14 (Fig. 1). The reaction was carried out in the format of a hydrolyzed probe using the following primers and probe: forward: 5'-TCACTCAGTGTTGAGCCACAG-3', reverse: 5'-TCCTGTGTTGGGTAGAAGTGG-3'; Universal Probe Library Probes Number 20 (Roche Molecular Systems). Using a 4 µL restriction enzyme-treated gDNA solution, each primer and probe were added to 10 µL of Essential Probe Master Mix (Roche Molecular Systems) at 0.4 µM, and analysis was performed in a total

volume of 20 µL. The cycling conditions included initial denaturation at 95 ℃ for 10 min, followed by cycles of 95 ℃ for 10 sec, 4.4 ℃/s, 60 ℃ for 30 sec, 2.2 ℃/s annealing. PCR was performed using the LightCycler 96 and data were analyzed using the LightCycler 96 software 1.1 (Roche Molecular Systems).

$$\mathrm{methylationpercentage}(\%) = 2^{\left(\Delta \mathrm{a} - \mathrm{Mcontrol}\Delta \mathrm{a}\right)} \times \left(1 - 2^{\left(\Delta \mathrm{b} - \mathrm{UMcontrol}\Delta \mathrm{b}\right)}\right) \times 100$$

$$\Delta \mathrm{a} = \mathrm{Cp}_{\mathrm{HapII}} - \mathrm{Cp}_{\mathrm{H2O}}$$

$$\Delta \mathrm{b} = \mathrm{Cp}_{\mathrm{MspI}} - \mathrm{Cp}_{\mathrm{H2O}}$$

The methylation percentage was calculated using the above formula. gDNA extracted from the T24 cell line was used as the unmethylated control, and EpiScope Methylated HeLa cell gDNA (Takara Bio) was used as the methylated control. The nucleic acid extraction solution was adjusted to concentrations of 0, 6.25, 12.5, 25, 50, and 100% and the reaction of the measurement system was confirmed. The methylation percentage was determined from the Cp value of each sample.

Assessment of mRNA expression via reverse transcription (RT)-qPCR

To investigate the correlation between *CALN1* methylation and mRNA expression, we performed an RT-qPCR-based assessment for the objective quantification of *CALN1* mRNA levels. Of the 82 cases, 19 that were quantitatively and qualitatively suitable for assays were used for this analysis. RNA was extracted from fresh frozen TURBT tissue using the High Pure RNA Isolation Kit (Roche Molecular Systems) according to the manufacturer's instructions. cDNA synthesis was performed under the following reaction conditions: 25 ℃ for 10 min, 55 ℃ for 60 min, and 85 ℃ for 5 min. The reaction product was diluted 5-fold with TE buffer and used for subsequent reactions. Primer sequences for *CALN1* and the internal reference gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)*, are shown in Table 1. RT-qPCR was carried out using the LightCycler 96 (Roche Molecular Systems), and the average value of duplicate measurements was determined using the LightCycler 96 software 1.1 (Roche Molecular Systems). The comparative C(T) method in relation to *GAPDH* was used for *CALN1* expression analysis, and the correlation between *CALN1* expression and the methylation percentage was analyzed.

Table 1
Primer sequencing

Genes	Forward primer	Reverse primer
CALN1	5'-GAAGGAGTGCATTCCCAGAA-3'	5'-GCTGCAATCAGCATGACACT-3'
GAPDH	5'-AGCCACATCGCTCAGACA-3'	5'-GCCCAATACGACCAAATCC-3'

Follow-up study

In our institute, cystoscopy is performed every three months after TURBT for the first two years, then every six months until five years. Intravesical recurrence of bladder cancer was defined as a tumor identified by cystoscopy and confirmed by pathological diagnosis. Intravesical BCG therapy after TURBT was performed at the discretion of the attending physician. Follow-up was conducted in November 2021. The time point of entry was defined as the date when TURBT data were obtained. The primary endpoint was the intravesical recurrence of bladder cancer.

Statistical analyses

Because the variables were non-normally distributed, they are expressed as medians and interquartile ranges. Differences between two groups were assessed using the Mann–Whitney U test. Fisher's exact test was used to analyze categorical variables. We used a Cox proportional hazards regression to examine the predictive value of *CALN1* methylation percentage for intravesical recurrence in NMIBC patients. The covariates included *CALN1* methylation percentage, age, sex, BCG therapy, stage, grade, number, size, and sample type (primary/recurrent). Baseline variables ($P < 0.05$) in the univariate analysis were included in the multivariate models. A receiver operating characteristic (ROC) curve was generated, and the area under the curve was calculated to determine the appropriate cut-off level of *CALN1* methylation percentage to maximize the predictive power for intravesical recurrence-free survival of NMIBC. The methylation percentage was grouped into low and high based on the cut-off value confirmed by the ROC curve analysis. Kaplan–Meier curves of estimated intravesical recurrence-free survival were generated, and comparisons between the groups were performed using a 2-sided log-rank test.

To assess whether the accuracy of predicting intravesical recurrence would improve after the addition of *CALN1* methylation percentage to established risk factors, including tumor stage, grade, number, size, and sample type, we calculated the C-index, net reclassification improvement, and integrated discrimination improvement. The *CALN1* methylation percentage and value of mRNA expression were not normally distributed (assessed by the Shapiro-Wilk test); therefore, non-parametric correlation coefficients (Spearman's [ρ]) were used to determine the association between *CALN1* methylation percentage and mRNA expression. Statistical significance was set at $P < 0.05$ and all statistical tests were two-sided. Statistical analyses were performed using the R software version 4.0.3.

Results

During the follow-up period (median, 11.5 months), 25 patients showed intravesical recurrence and 13 died. The median *CALN1* methylation percentage was 79.5% (interquartile range: 51.1, 92.6). In the univariate Cox proportional hazards analysis, *CALN1* methylation percentage was a significant predictor of intravesical recurrence (hazard ratio (HR) 0.98, 95% confidence interval (CI); 0.97–1.00, $P = 0.0010$). After adjustment for other confounders, *CALN1* methylation percentage was an independent predictor of intravesical recurrence (HR 0.98, 95% CI; 0.97–1.00, $P = 0.0177$). ROC analysis was performed to maximize the predictive power of *CALN1* methylation percentage for intravesical recurrence, and the 87% cut-off value was obtained (area under the curve = 0.711). Based on the cut-off value, 82 patients fit into two groups. Fifty-one (62%) patients fit in the low group with a methylation percentage of less than 87%,

and 31 (38%) fit in the high group with a methylation percentage greater than 87%. Patient characteristics according to *CALN1* methylation percentage are shown in Table 2. Of the 82 cases, 73 were NMIBC and nine were MIBC. Total cystectomy was performed in two NMIBC cases that were difficult to cure by TURBT during the follow-up period. Patients in the low group were significantly older than those in the high group and had a higher proportion of females and non-smokers. In addition, the low group tended to have significantly advanced tumor stages and more histologically high-grade tumors than the high group.

Table 2
Clinicopathological features according to CALN1 methylation percentage

Variables		All	*CALN1* methylation		P
		n = 82	Low	High	
			n = 51	n = 31	
CALN1 methylation percentage (IQR)		79.5 (51.1, 92.6)	63.1 (40.7, 78.1)	97.8 (90.6, 100)	< .0001
Follow-up period, month (range)		11.5 (0–29)	12 (0–29)	11 (0–29)	0.208
Age (range)		76 (52–93)	79 (53–93)	73 (52–85)	< .0001
Gender	female, n (%)	19 (23)	17 (33)	2 (6)	0.0062
Smoking	yes, n (%)	45 (55)	23 (45)	22 (71)	0.0386
BCG[b] therapy	yes, n (%)	11 (13)	7 (13)	4 (13)	1
Total cystectomy		9 (11)	5 (10)	4 (13)	0.7238
Tumor stage, n (%)					0.0007
	Ta	54 (66)	27 (53)	27 (87)	
	T1	19 (23)	18 (35)	1 (3)	
	T2+	9 (11)	6 (12)	3 (10)	
Tumor grade, n (%)					0.0178
	Low	62 (76)	34 (67)	28 (90)	
	High	20 (24)	17 (33)	3 (10)	
Tumor number, n (%)					0.6491
	Single	41 (50)	24 (47)	17 (55)	
	Multiple	41 (50)	27 (53)	14 (45)	
Tumor size, n (%)					0.1211
	< 30mm	70 (85)	41 (80)	29 (94)	
	≥ 30mm	12 (15)	10 (20)	2 (6)	
Sample type, n (%)					0.6475
	Primary	50 (61)	30 (59)	20 (65)	
	Recurrent	32 (39)	21 (41)	11 (35)	

[a] IQR, interquartile range; [b] BCG; Bacillus Calmette-Guérin

To identify the association between *CALN1* methylation percentage and intravesical recurrence, a Kaplan–Meier analysis was performed for 71 NMIBC cases where the bladder was preserved. There was a significant difference between the two groups in intravesical recurrence-free survival (P = 0.0084). At the one-year follow-up, the Kaplan–Meier survival rates for intravesical recurrence were 48.2% and 86.3% in the low and high groups, respectively (Fig. 2). The results of univariate and multivariate Cox regression analyses to explore the prognostic factors of intravesical recurrence are shown in Table 3. A low *CALN1* methylation percentage remained an independent prognostic factor after adjustment for tumor size in the multivariate analysis. The C-index increased, but did not reach statistical significance (0.744, P = 0.268). However, the net reclassification improvement and integrated discrimination improvement for the intravesical recurrence rate significantly improved after adding *CALN1* methylation percentage into the baseline model with established risk factors (0.57 and 0.07, P = 0.021 and P = 0.025, respectively, Table 4). In the analysis of the correlation between *CALN1* methylation and the mRNA expression level, a significant negative correlation was observed (Fig. 3).

Table 3
Prognostic value of CALN1 methylation percentage for intravesical recurrence of bladder cancer

Variables	Univariate		Multivariate	
	HR[a] (95% CI[b])	P	HR (95% CI)	P
CALN1 methylation percentage	0.98 (0.97-1.00)	0.0107	0.98 (0.97-1.00)	0.0177
Age	1.03 (0.99–1.09)	0.1536		
Gender (male)	0.63 (0.25–1.60)	0.3343		
BCG[c] therapy (yes)	0.41 (0.10–1.75)	0.2292		
Stage (Ta)	0.56 (0.24–1.32)	0.1902		
Grade (low)	0.63 (0.25–1.61)	0.3417		
Number (single)	1.02 (0.47–2.26)	0.9503		
Size (≥ 30mm)	4.25 (1.50–12.1)	0.0065	3.75 (1.33–10.6)	0.0123
Recurrent tumor	0.84 (0.38–1.88)	0.6825		

[a] HR; Hazard ratio, [b] CI; Confidence interval, [c] BCG; Bacillus Calmette-Guérin

Table 4
Discrimination of each predictive model for intravesical recurrence using C-index, net reclassification improvement (NRI), and integrated discrimination improvement (IDI)

Predictive models	C-index	P-Value	NRI	P-Value	IDI	P-Value
Established risk factors[a]	0.67 (0.53–0.82)	Reference	Reference		Reference	
+*CALN1* methylation percentage	0.74 (0.61–0.87)	0.268	0.57	0.0214	0.07	0.0247

[a] Established risk factors included tumor stage, grade, number, size, and sample type.

Discussion

We analyzed the relationship between *CALN1* methylation percentage and clinicopathological data of bladder cancer patients. We found that there exists a significant association between a lower *CALN1* methylation percentage and advanced tumor stage and histologically grade and a significantly increased risk of intravesical recurrence. To the best of our knowledge, this is the first study to show that *CALN1* methylation percentage is associated with the clinicopathological features and prognosis of bladder cancer.

DNA methylation is considered to be an epigenetic modification, and its association with many biological phenomena, including carcinogenesis, has been established [13, 14]. Methylation analyses have also found some information (e.g., drug susceptibility [15], prognosis prediction, and risk [16]) that cannot be obtained with conventional test data.

Cao et al. used microarray analysis to show that calcium signal transduction was associated with the development of bladder cancer via the mitogen-activated protein kinase pathway [17]. We hypothesized that the regulation of calcium signal transduction through methylation of *CALN1* was involved in the development and progression of bladder cancer. In addition, intron 2 of *CALN1* is a DNase I hypersensitive site that is strongly associated with transcriptional activity [18]. Therefore, we suspected that *CALN1* methylation was involved in the action of a DNase I hypersensitive site and, as a result, may affect the expression of *CALN1*. Regarding the relationship between bladder cancer and methylation, various analytical reports have centered on CpG sites [19, 20], and testing systems such as Bladder EpiCheck [21] have been established. Although various trials have been conducted regarding the diagnosis and treatment of bladder cancer, methylation analysis of *CALN1* and its association with bladder cancer has not been probed before.

Bisulfite sequencing is widely used for methylation analyses. In this study, we performed methylation analysis using methylation-sensitive restriction enzyme (MSRE)-qPCR. This technique enables the analysis of a small amount of sample obtained by TURBT without bisulfite treatment. Bisulfite treatment involves mixing the corrosive chemical bisulfite salt with DNA and heating it to 50–70 ℃. It is known that

DNA is cleaved in this process, and the yield is extremely low. Recently, high-yield methods have been developed, but fragmentation has not been completely avoided [22]. In addition, because bisulfite sequencing requires a large number of cells, it is not feasible when a small amount of DNA is available from clinical specimens, such as cell-free DNA and circulating tumor cells. In contrast, one of the advantages of MSRE-qPCR is the side-by-side comparison between control and experimental samples, even for very low amounts of DNA. In addition, MSRE-qPCR can be completed in less time than other methods with the same level of accuracy [23]. Comprehensive analysis using next-generation sequencing is also useful but less practical owing to high costs. MSRE-qPCR is useful for targeted analysis owing to its simple workflow. Further investigation exploring this diagnostic method with high sensitivity and specificity in combination with other diagnostic markers is necessary and will contribute to the development of new diagnostic systems for bladder cancer.

The current study has some limitations. First, there were no criteria for intravesical BCG immunotherapy, though there was no difference in BCG therapy between the low- and high-methylation groups. Second, the sample size was small and the follow-up period was short. Therefore, the findings of this study need to be validated in a larger study.

Conclusions

We performed methylation analysis of intron 2 of *CALN1* using genomic DNA extracted from samples collected by TURBT. We found that low *CALN1* methylation percentage is consistent with the occurrence of advanced tumor stages, high-grade tumors, and higher intravesical recurrence rates. Therefore, we suggest that *CALN1* methylation percentage may be an indicator of high-risk bladder cancer and could be considered a useful biomarker for accurately predicting intravesical recurrence of NMIBC.

Abbreviations

MSRE: Methylation-sensitive restriction enzyme

qPCR: Quantitative polymerase chain reaction

MIBC: Muscle-invasive bladder cancer

TURBT: Transurethral resection of bladder tumor

gDNA: Genomic DNA

RT-qPCR: Reverse transcription-qPCR

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

ROC: Receiver operating characteristic

HR: Hazard ratio

CI: Confidence interval

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Shakai Iryo Hojin Daiyukai (approval number: 2019002) and written informed consent was obtained from all patients.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare no conflict of interest.

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Author's contributions

AK and AN conceived the idea of the study. KT developed the statistical analysis plan and conducted statistical analyses. KT and TK contributed to the interpretation of the results. KT drafted the original manuscript. AK and MH supervised the conduct of this study. KA, YM, and KK contributed to data curation. All authors reviewed the manuscript draft and revised it critically on intellectual content. All authors approved the final version of the manuscript to be published.

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References

1. van Rhijn BW, Burger M, Lotan Y, et al. Recurrence and progression of disease in non-muscle-invasive bladder cancer: from epidemiology to treatment strategy. Eur Urol. 2009;56:430–42.

doi:10.1016/j.eururo.2009.06.028.

2. Pajor G, Somogyi L, Melegh B, et al. Urovysion: considerations on modifying current evaluation scheme, including immunophenotypic targeting and locally set, statistically derived diagnostic criteria. Cytometry A. 2011;79:375–82. doi:10.1002/cyto.a.21065.
3. Raitanen MP, Marttila T, Nurmi M, et al. Human complement factor H related protein test for monitoring bladder cancer. J Urol. 2001;165:374–7. doi:10.1097/00005392-200102000-00005.
4. Landman J, Chang Y, Kavaler E, et al. Sensitivity and specificity of NMP-22, telomerase, and BTA in the detection of human bladder cancer. Urology. 1998;52:398–402. doi:10.1016/s0090-4295(98)00219-2.
5. Pajor G, Sule N, Alpar D, et al. Increased efficiency of detecting genetically aberrant cells by UroVysion test on voided urine specimens using automated immunophenotypical preselection of uroepithelial cells. Cytometry A. 2008;73:259–65. doi:10.1002/cyto.a.20528.
6. Robertson AG, Kim J, Al-Ahmadie H, et al. Comprehensive molecular characterization of muscle-invasive bladder Cancer. Cell. 2017;171:540–56.e25. doi:10.1016/j.cell.2017.09.007.
7. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med. 2005;352:997–1003. doi:10.1056/NEJMoa043331.
8. deVos T, Tetzner R, Model F, et al. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. Clin Chem. 2009;55:1337–46. doi:10.1373/clinchem.2008.115808.
9. Queirós AC, Villamor N, Clot G, et al. A B-cell epigenetic signature defines three biologic subgroups of chronic lymphocytic leukemia with clinical impact. Leukemia. 2015;29:598–605. doi:10.1038/leu.2014.252.
10. Wu YQ, Lin X, Liu CM, et al. Identification of a human brain-specific gene, calneuron 1, a new member of the calmodulin superfamily. Mol Genet Metab. 2001;72:343–50. doi:10.1006/mgme.2001.3160.
11. Roderick HL, Cook SJ. Ca2 + signalling checkpoints in cancer: remodelling Ca2 + for cancer cell proliferation and survival. Nat Rev Cancer. 2008;8:361–75. doi:10.1038/nrc2374.
12. Shapovalov G, Ritaine A, Skryma R, et al. Role of TRP ion channels in cancer and tumorigenesis. Semin Immunopathol. 2016;38:357–69. doi:10.1007/s00281-015-0525-1.
13. Laird PW. The power and the promise of DNA methylation markers. Nat Rev Cancer. 2003;3:253–66. doi:10.1038/nrc1045.
14. Bock C. Epigenetic biomarker development. Epigenomics. 2009;1:99–110. doi:10.2217/epi.09.6.
15. Ohmura H, Ito M, Uchino K, et al. Methylation of drug resistance-related genes in chemotherapy-sensitive Epstein–Barr virus-associated gastric cancer. FEBS Open Bio. 2020;10:147–57. doi:10.1002/2211-5463.12765.
16. Yu Y, Cao H, Zhang M, et al. Prognostic value of DNA methylation for bladder cancer. Clin Chim Acta. 2018;484:207–12. doi:10.1016/j.cca.2018.05.056.
17. Cao R, Meng Z, Liu T, et al. Decreased TRPM7 inhibits activities and induces apoptosis of bladder cancer cells via ERK1/2 pathway. Oncotarget. 2016;7:72941–60. doi:10.18632/oncotarget.12146.

18. Wang YM, Zhou P, Wang LY, et al. Correlation between DNase I hypersensitive site distribution and gene expression in HeLa S3 cells. PLoS ONE. 2012;7:e42414. doi:10.1371/journal.pone.0042414.
19. Guo RQ, Xiong GY, Yang KW, et al. Detection of urothelial carcinoma, upper tract urothelial carcinoma, bladder carcinoma, and urothelial carcinoma with gross hematuria using selected urine-DNA methylation biomarkers: A prospective, single-center study. Urol Oncol. 2018;36:342.e15–23. doi:10.1016/j.urolonc.2018.04.001.
20. Reinert T, Modin C, Castano FM, et al. Comprehensive genome methylation analysis in bladder cancer: identification and validation of novel methylated genes and application of these as urinary tumor markers. Clin Cancer Res. 2011;17:5582–92. doi:10.1158/1078-0432.CCR-10-2659.
21. van der Heijden AG, Mengual L, Ingelmo-Torres M, et al. Urine cell-based DNA methylation classifier for monitoring bladder cancer. Clin Epigenet. 2018;10:71. doi:10.1186/s13148-018-0496-x.
22. Millar D, Christova Y, Holliger P. A polymerase engineered for bisulfite sequencing. Nucleic Acids Res. 2015;43:e155. doi:10.1093/nar/gkv798.
23. Perry N, Wasko K, Cheng J, et al. Methylation-sensitive restriction enzyme quantitative polymerase chain reaction enables rapid, accurate, and precise detection of methylation status of the regulatory T cell (Treg)-specific demethylation region in primary human Tregs. J Immunol. 2021;206:446–51. doi:10.4049/jimmunol.1901275.

Figures

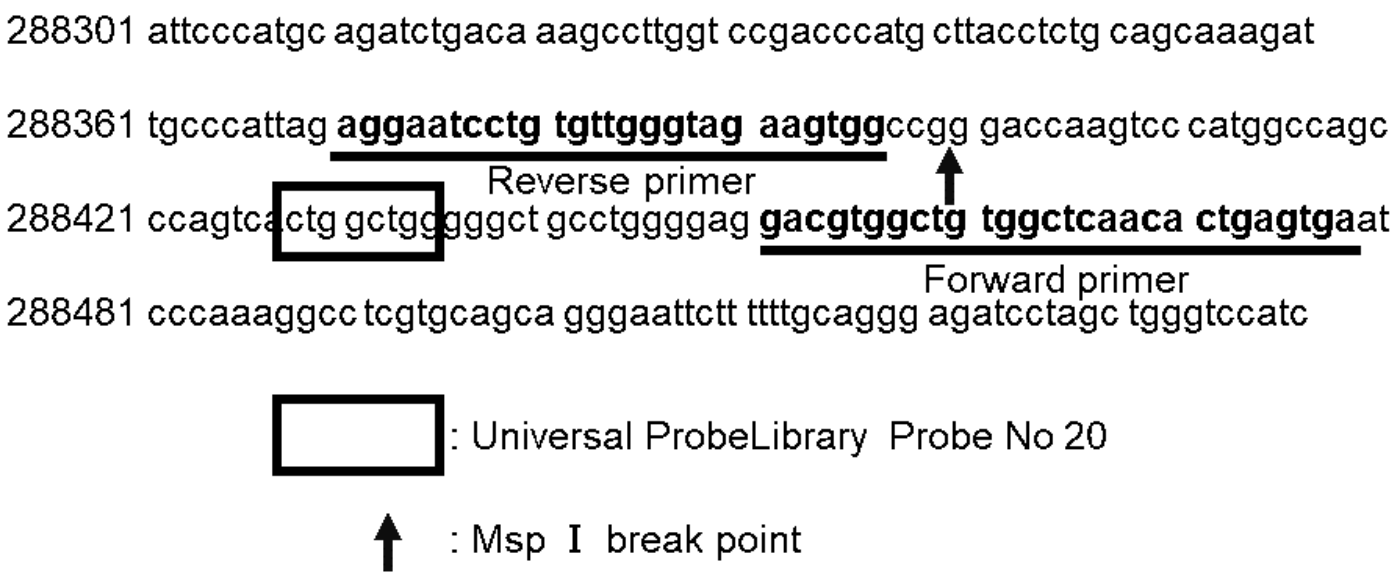

Figure 1

The primers were designed using the intron 2 sequence of *CALN1* with the GenBank accession no. NC_000007.14

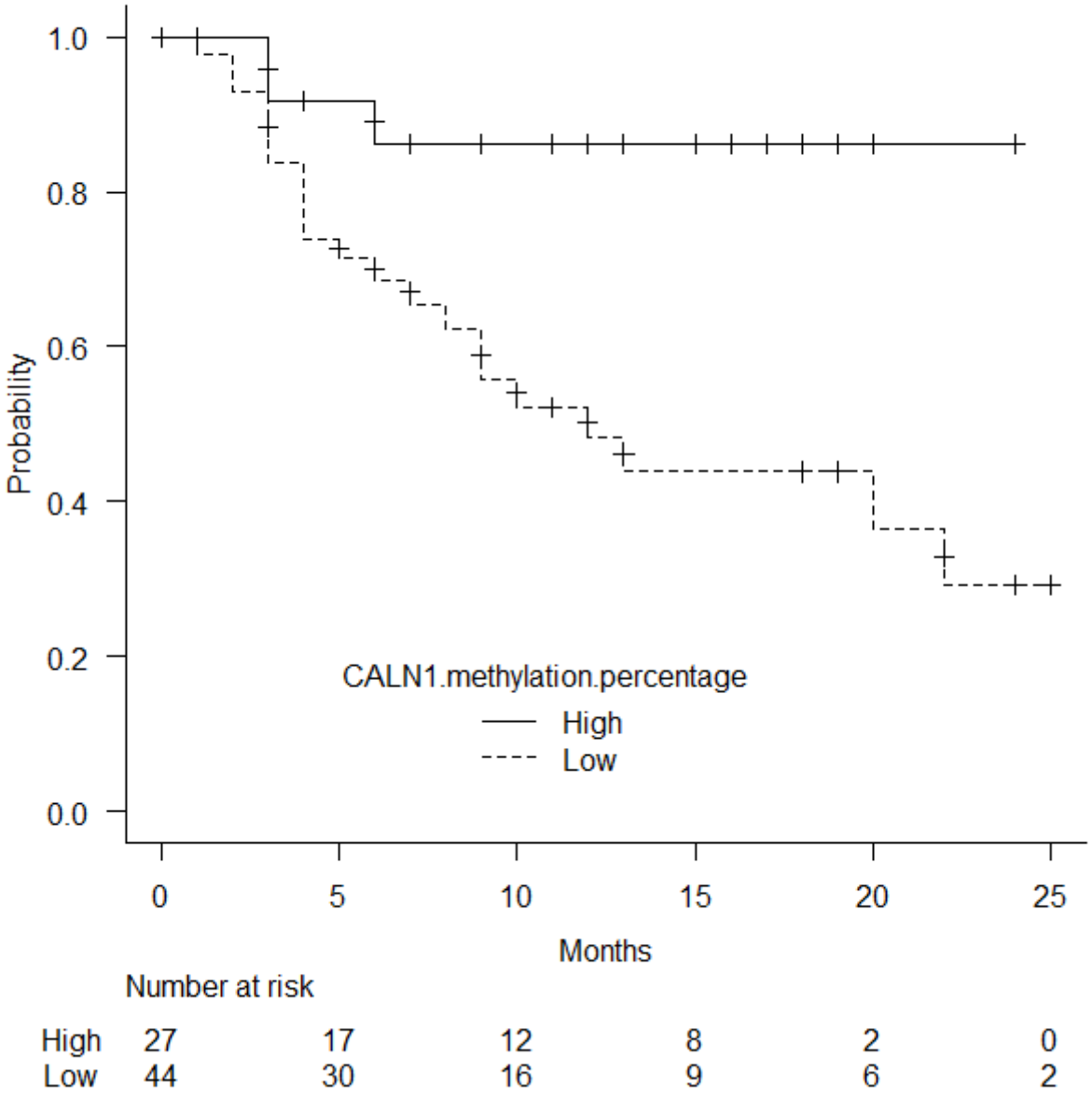

Figure 2

Kaplan-Meier curves of intravesical recurrence-free survival of NMIBC patients in high and low *CALN1* methylation percentages

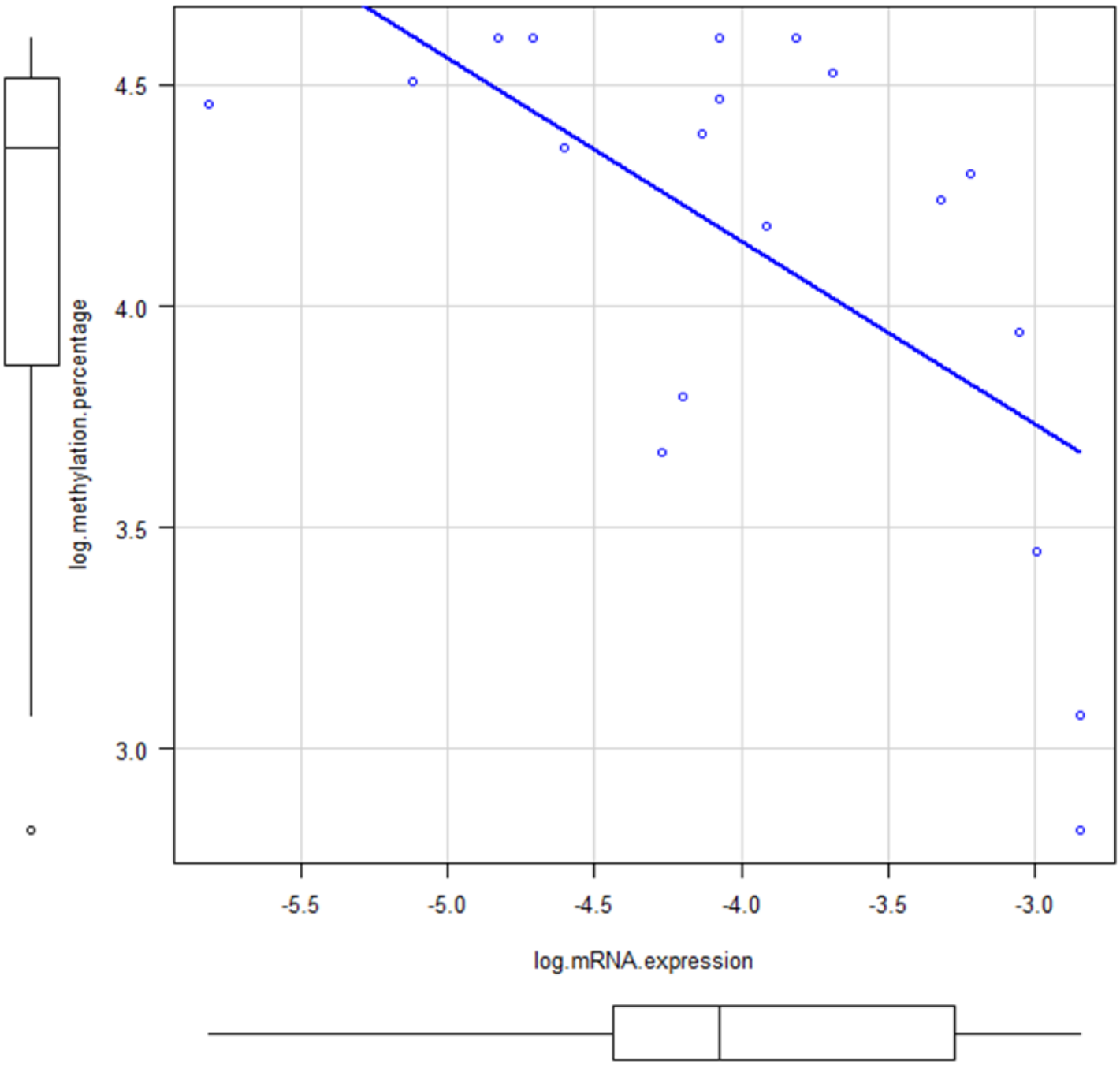

Figure 3

CALN1 methylation percentage has a negative relationship with *CALN1* mRNA expression (Spearman's ρ = -0.563 and P = 0.0121)

Supplementary Files

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- Supplementalfile1.xlsx