Supplementary Materials: Diagnostic and prognostic value of *B4GALT1* hypermethylation and its clinical significance as a novel circulating cell-free DNA biomarker in colorectal cancer

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Table S1. Patients and samples selection of metastatic colorectal cancer.

Type of Specimens	Group 1 [a] (Training Set)	Group 2 [b] (Validation Set)	Group 3T [c] (Training Set)	Group 3V [c] (Validation Set)	Group 4 [d] (Controls)
Patients	27	22	25	26	19
Tumor Tissue	24/27	15/22	25/25		
Normal Tissue	22/27				
Metastasis Tissue	27/27	22/22	1/25		
Normal tissue surrounding the metastasis		15/22			
Plasma			20/25	26/26	19/19
Analysis performed	QMSP [e]	QMSP	QMSP + dd-QMSP [f]	dd-QMSP	dd-QMSP

[a] Group 1: metastatic colorectal cancer patients from University Campus Bio-Medico of Rome (UCBM). [b] Group 2: metastatic colorectal cancer patients from "Ospedale Casa Sollievo della Sofferenza" (IRCCS-CSS). [c] Group 3T and 3V: metastatic colorectal cancer patients from University Hospital of Santiago de Compostela (CHUS). [d] Group 4: healthy controls from the "Lazzaro Spallanzani" National Institute for infectious Diseases (INMI) of Rome, and from University Hospital of Santiago de Compostela (CHUS). [e] QMSP: Quantitative-Methylation-Specific-PCR. [f] dd-QMSP: Droplet-Digital-Quantitative-Methylation-Specific-PCR.

Table S2. Clinical characteristics of 1418 patients in cohort 1 to.6 stratified by expression level of *B4GALT1*.

Cohort 1 (n = 226)			
Variable	*B4GALT1* low	*B4GALT1* high	p-value
Gender [a]	N = 182 (80.5%)	N = 44 (19.5%)	0.6471
Male	98 (53.8%)	22 (50%)	
Female	84 (46.2%)	22 (50%)	
Age (mean ± SD) [b]	65.72 ± 13.35	67.32 ± 11.58	0.4653
Stage [a]	N = 182 (80.5%)	N = 44 (19.5%)	0.0300
I	31 (17%)	10 (22.7%)	
II	70 (38.5%)	24 (54.5%)	
III	81 (44.5%)	10 (22.7%)	
Location [a]	N = 180 (80.4%)	N = 44 (19.6%)	0.0874
Proximal R	75 (41.7%)	26 (59.1%)	
Distal L	78 (43.3%)	15 (34.1%)	
Rectum	27 (15%)	3 (6.8%)	
Cohort 2 (n = 130)			

Gender [a]	*N* = 60 (46.2%)	*N* = 70 (53.8%)	0.9569
Male	32 (53.3%)	37 (52.9%)	
Female	28 (46.7%)	33 (47.1%)	
Age (mean ± SD) [b]	69.28 ± 12.69	67.37 ± 12.71	0.3938
Stage [a]	*N* = 60 (46.2%)	*N* = 70 (53.8%)	<0.0001
I	0 (0%)	0 (0%)	
II	20 (33.3%)	53 (75.7%)	
III	40 (66.7%)	17 (24.3%)	
Location [a]	*N* = 59 (45.7%)	*N* = 70 (54.3%)	0,7045
Proximal R	25 (42.4%)	32 (45.7%)	
Distal L	34 (57.6%)	38 (54.3%)	
Rectum	0 (0%)	0 (0%)	
Cohort 3 (*n* = 557)			
Gender [a]	*N* = 206 (37%)	*N* = 351 (63%)	0.2548
Male	120 (58.3%)	187 (53.3%)	
Female	86 (41.7%)	164 (46.7%)	
Age (mean ± SD)[b]	66.49 ± 13.35	67.06 ± 13.28	0.6276
Stage[a]	*N* = 206 (37%)	*N* = 351 (63%)	0.0086
I	13 (6.3%)	23 (6.6%)	
II	78 (39.7%)	182 (51.9%)	
III	86 (41.7%)	115 (32.8%)	
IV	29 (14.1%)	31 (8.8%)	
Location [a]	*N* = 206 (37%)	*N* = 351 (63%)	0.0015
Proximal R	63 (30.6%)	155 (44.2%)	
Distal L	143 (69.4%)	196 (55.8%)	
Rectum	0 (0%)	0 (0%)	
KRAS status [a]	*N* = 194 (36.2%)	*N* = 342 (63.8%)	0.0532
Wild type	106 (51.5%)	216 (61.5%)	
Mutant	88 (42.7%)	126 (35.9%)	
Cohort 4 (*n* = 125)			
Gender [a]	*N* = 23 (18.4%)	*N* = 102 (81.6%)	0.2333
Male	9 (39.1%)	54 (52.9%)	
Female	14 (60.9%)	48 (47.1%)	
Age (mean ± SD) [b]	64.26 ± 12.48	65.02 ± 13.75	0.8084
Stage [a]	*N* = 23 (18.4%)	*N* = 102 (81.6%)	0.0004
I	4 (17.4%)	24 (23.5%)	
II	2 (8.7%)	46 (45.1%)	
III	17 (73.9%)	32 (31.4%)	
IV	0 (0%)	0 (0%)	
Location [a]	*N* = 23 (18.4%)	*N* = 102 (81.6%)	0.8523
Proximal R	8 (34.8%)	42 (41.2%)	
Distal L	13 (56.5%)	52 (51%)	
Rectum	2 (8.7%)	8 (7.8%)	
Cohort 5 (*n* = 80)			
Gender [a]	*N* = 44 (55%)	*N* = 36 (45%)	0.3234
Male	22 (50%)	22 (61.1%)	
Female	22 (50%)	14 (38.9%)	
Age (mean ± SD) [b]	61.26 ± 12.33	60.39 ± 14.36	0.7734
KRAS status [a]	*N* = 36 (51.4%)	*N* = 34 (48.6%)	0.6657
WT	23 (52.3%)	20 (55.6%)	
Mutant	13 (29.5%)	14 (38.9%)	
Cohort 6 TCGA (*n* = 300)			
Gender [a]	*N* = 35 (11.6%)	*N* = 265 (88.4%)	0.4673
Male	17 (48.6%)	146 (55.1%)	
Female	18 (51.4%)	119 (44.9%)	
Age (mean ± SD) [b]	67.23 ± 13.45	65.19 ± 13.07	0.3888
Stage [a]	*N* = 34 (11.3%)	*N* = 256 (88.7%)	0.6082
I	3 (8.6%)	46 (17.2%)	

II	15 (42.9%)	103 (38.6%)	
III	11 (31.4%)	76 (28.5%)	
IV	5 (14.3%)	31 (11.6%)	
KRAS status [a]	*N* = 6 (2%)	*n* = 33 (98%)	0.8393
WT	3 (8.6%)	18 (6.7%)	
Mutant	3 (8.6%)	15 (5.6%)	

[a] χ^2-test. [b] *t*-test.

Table S3. Demographic and clinicopathological characteristics of colorectal cancer cases

Variable	Group 1	Group 2	Group 3T+ 3V
Total Patients	27/27	8/22 [a]	54/54
	Gender		
Male	19 (70,4%)	7 (87,5%)	17 (31,4%)
Female	8 (29,6%)	1 (12,5%)	37 (68,5%)
	Age		
Median (Range)	68,5 (42–79)	63 (47–84)	64 (38–93)
	Tumor site		
Rectum	3 (11,1%)	2 (25%)	18 (33,3%)
Sigmoid colon	2 (7,4%)	2 (25%)	19 (35,1%)
Descending colon	5 (18,5%)	1 (12,5%)	1 (1,8%)
Transverse colon	0 (0%)	0 (0%)	7 (12,9%)
Ascending colon	17 (63%)	3 (37,5%)	9 (16,6%)
	Histologic type		
Adenocarcinoma moderately differentiated	25 (92,6%)	8 (100%)	53 (98,1%)
Infiltrating adenocarcinoma	2 (7,4%)	0 (0%)	1 (1,9%)
	Grading		
G1	0 (0%)	0 (0%)	14 (25,9%)
G2	21 (77,8%)	3 (37,5%)	22 (40,7%)
G3	5 (18,5%)	0 (0%)	6 (11,1%)
undetermined	1 (3,7%)	5 (62,5%)	12 (22,2%)
	Pathologic tumor stage		
T1	1 (3,7%)	0 (0%)	0 (0%)
T2	2 (7,4%)	0 (0%)	2 (3,7%)
T3	17 (63%)	2 (25%)	30 (55,5%)
T4	7 (25,9%)	0 (0%)	11 (20,3%)
X		6 (75%)	11 (20,3%)
	Pathologic nodal stage		
n+	18 (66,7%)	1 (12,5%)	35 (64,8%)
n−	9 (33,3%)	3 (37,5%)	5 (9,2%)
X		4 (50%)	14 (25,9%)
	Site of Metastasis [b]		
Liver	27 (90%)	4 (40%)	43 (55,8%)
Lung	0 (0%)	2 (20%)	17 (22%)
Peritoneum	1 (3,3%)	1 (10%)	8 (10,3%)
Other	2 (6,7%)	1 (10%)	9 (11,6%)
	UICC stage [c]		
I	2 (7,4%)	0 (0%)	0 (0%)
II A	3 (11,1%)	2 (25%)	0 (0%)
II B	0 (0%)	0 (0%)	0 (0%)
III A	1 (3,7%)	0 (0%)	0 (0%)
III B	0 (0%)	1 (12,5%)	0 (0%)
III C	2 (7,4%)	0 (0%)	0 (0%)
IV	19 (70,4%)	5 (62,5%)	54 (93%)
	Alcohol		
No	0 (0%)	0 (0%)	7 (12,9%)
Yes	0 (0%)	0 (0%)	10 (18,5%)

undetermined	27 (100%)	8 (100%)	37 (68,5%)
	Smoke		
No	0 (0%)	0 (0%)	11 (20,3%)
Yes	0 (0%)	0 (0%)	10 (18,5%)
undetermined	27 (100%)	8 (100%)	33 (61,1%)
	KRAS mutation		
WT [d]	0 (0%)	4 (50%)	30 (55,5%)
Codon 12/13 exon 2	0 (0%)	0 (0%)	5 (9,2%)
Codon 12	1 (3,7%)	3 (37,5%)	8 (14,8%)
Codon 13	0 (0%)	1 (12,5%)	5 (9,2%)
Codon 61	0 (0%)	0 (0%)	1 (1,8%)
undetermined	26 (96,3%)	0 (0%)	5 (9,2%)
	BRAF mutation		
WT [d]	1 (3,7%)	0 (0%)	1 (1,9%)
V600E exon 15	0 (0%)	0 (0%)	0 (0%)
undetermined	26 (96,3%)	8 (100%)	53 (98,1%)
	MSI status		
MSS	0 (0%)	0 (0%)	7 (13%)
MSI	0 (0%)	0 (0%)	0 (0%)
undetermined	27 (100%)	8 (100%)	47 (87%)

[a] Group 2: the Demographic and clinicopathological information were available for 8/22 cases. [b] Some patient had more than one metastasis at different sites. [c] UICC: Union for International Cancer Control. [d] WT: wild type.

Table S4. Univariate and multivariate analysis of factors affecting DFS in stage I-III patients (data from GEO cohorts 1, 2 and 3 were pooled together $n = 853$).

		Univariate analysis [a]		**Multivariate analysis** [b]	
Variables	***n***	**5-Years DFS**	***p*-value**	**HR (95% CI)**	***p*-value**
<70 y	447	68.50%	-	-	-
≥70 y	405	74.60%	0.1517	-	-
Female	388	75.20%	0.0760	0.7725 (0.57–1.03)	0. 0835
Male	465	68.00%	-	1 (-)	-
Left	462	69.30%	-	-	-
Rectum	30	77.50%	-	-	-
Right	358	73.30%	-	-	-
Done	289	64.40%	-	1.0133 (0.72–1.42)	0.9395
Undone	433	77.30%	-	1 (-)	-
I	77	95.40%	-	0.1758 (0.05–0.55)	0.0033
II	427	79.10%	-	1 (-)	-
III	349	57.10%	-	2.0515 (1.45–2.90)	0.0001
High	434	75.80%	0.0038	1 (-)	-
Low	419	66.50%	-	1.2133 (0.91–1.62)	0.1938

[a] In univariate analyses, log-rank tests were conducted. [b] In the multivariate Cox proportional hazard model, only variables with $p < 0.15$ in univariate analysis were included and the "enter method" was applied. [c] Data on age of GSM972293 of cohort 3 was not specified.

Table S5. *KRAS* mutational status and response to cetuximab monotherapy according to *B4GALT1* expression status in GEO cohort 5 (n = 80).

(**A**) Disease control rate versus *B4GALT1* expression (n = 80; p = 0.0708)		
Heading Title	*B4GALT1* High (36)	*B4GALT1* Low (44)
CR/PR/SD	15 (41.7%)	10 (22.7 %)
PD/UTD	21 (58.3%)	34 (77.3%)

All 80 patients were included in this analysis. Abbreviations: CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease, UTD, undetermined.

(**B**) Disease control rate versus *B4GALT1* expression (n = 68/80; p = 0.1056)		
Heading Title	*B4GALT1* High (32)	*B4GALT1* Low (36)
CR/PR/SD	15 (46.9%)	10 (27.8%)
PD	17 (53.1%)	26 (72.2%)

68/80 patients were included and 12/80 patients without response data (UTD, undetermined) were excluded in this analysis. Abbreviations: CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease.

(**C**) Response rate versus *B4GALT1* expression (in *KRAS* WT patients n = 43/80; p = 0.5250)		
Heading Title	*B4GALT1* High (20)	*B4GALT1* Low (23)
PR	3 (15%)	2 (8.7 %)
SD/PD/UTD	17 (85%)	21 (91.3%)

43/80 patients with *KRAS* WT were included in this analysis. Abbreviations: PR, partial remission; SD, stable disease; PD, progressive disease, UTD, undetermined; WT, wild-type.

(**D**) Response rate versus *B4GALT1* expression (in *KRAS* WT patients n = 39/80; p = 0.680)		
Heading Title	*B4GALT1* High (20)	*B4GALT1* Low (19)
PR	3 (15%)	2 (10.5 %)
SD/PD	17 (85%)	17 (89.5%)

39/80 patients with *KRAS* WT were included in this analysis, 4/80 patients with *KRAS* WT who had no response data (UTD) were excluded. Abbreviations: PR, partial remission; SD, stable disease; PD, progressive disease, WT, wild-type.

(**E**) Disease control rate versus *B4GALT1* expression (in *KRAS* WT patients n= 43/80; p = 0.0251)		
Heading Title	*B4GALT1* High (20)	*B4GALT1* Low (23)
PR/SD	13 (65%)	7 (30.4 %)
PD/UTD	7 (35%)	16 (69.6%)

43/80 patients with *KRAS* WT were included in this analysis. Abbreviations: PR, partial remission; SD, stable disease; PD, progressive disease; UTD, undetermined WT, wild-type.

(**F**) Disease control rate versus *B4GALT1* expression (in *KRAS* WT patients n = 39; p = 0.0826)		
Heading Title	*B4GALT1* High (20)	*B4GALT1* Low (19)
PR/SD	13 (65%)	7 (36.8%)
PD	7 (35%)	12 (63.2%)

39/80 patients with *KRAS* WT were included in this analysis, 4/80 patients with *KRAS* WT who had no response data (UTD) were excluded. Abbreviations: PR, partial remission; SD, stable disease; PD, progressive disease, WT, wild-type.

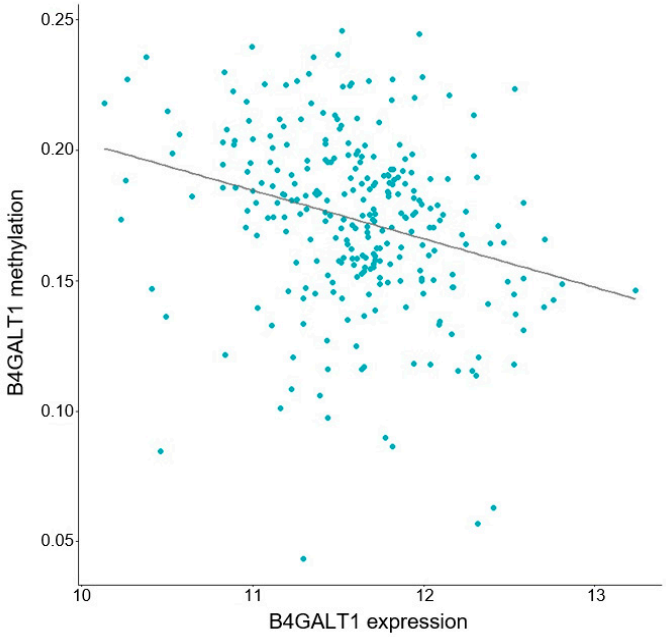
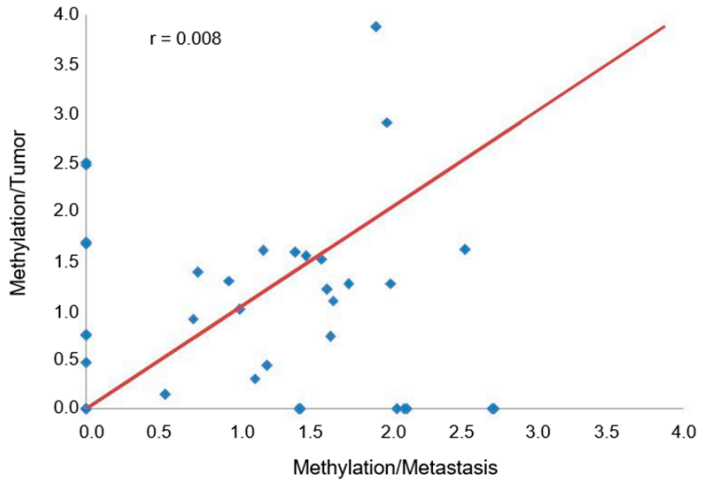


Figure 1. Inverse correlation of *B4GALT1* mRNA expression with the DNA methylation status in cohort 6 (TCGA-COAD). Spearman correlation coefficient = −0.3018, $p = 0.0001$.

A



B

		Metastasis Methylation	
Tumor Methylation	**Methylated**	**Unmethylated**	**Total**
Methylated	19/37 (51%)	7/37 (19%)	26 (70%)
Unmethylated	7/37 (19%)	4/37 (11%)	11 (30%)
Total	26 (70%)	11 (30%)	37 (100%)

Figure 2. Concordance analysis between tumor and matched metastases in CRC (Group 1 and 2, $n = 37$). (**A**) The concordance analysis was conducted considering the methylation continuous data. Concordance correlation coefficient (r) = 0.008 ($r \leq 0.90$ indicates poor degree of concordance; $r = 0.90$–0.95 indicates moderate degree of concordance; r = 0.95–0.99 indicates substantial degree of concordance; $r > 0.99$ indicates almost perfect degree of concordance). (**B**) The concordance analysis was conducted considering the methylation status as categorical data based on the cut-off defined by the presence of methylation in normal colon tissue. Concordance correlation coefficient (r) = 0.095 ($r \leq 0.20$ indicates poor degree of concordance; $r = 0.21$–0.40 indicates fair degree of concordance; $r = 0.41$–0.60 indicates moderate degree of concordance; $r = 0.61$–0.80 indicates good degree of concordance; $r = 0.81$–1 indicates very good degree of concordance).

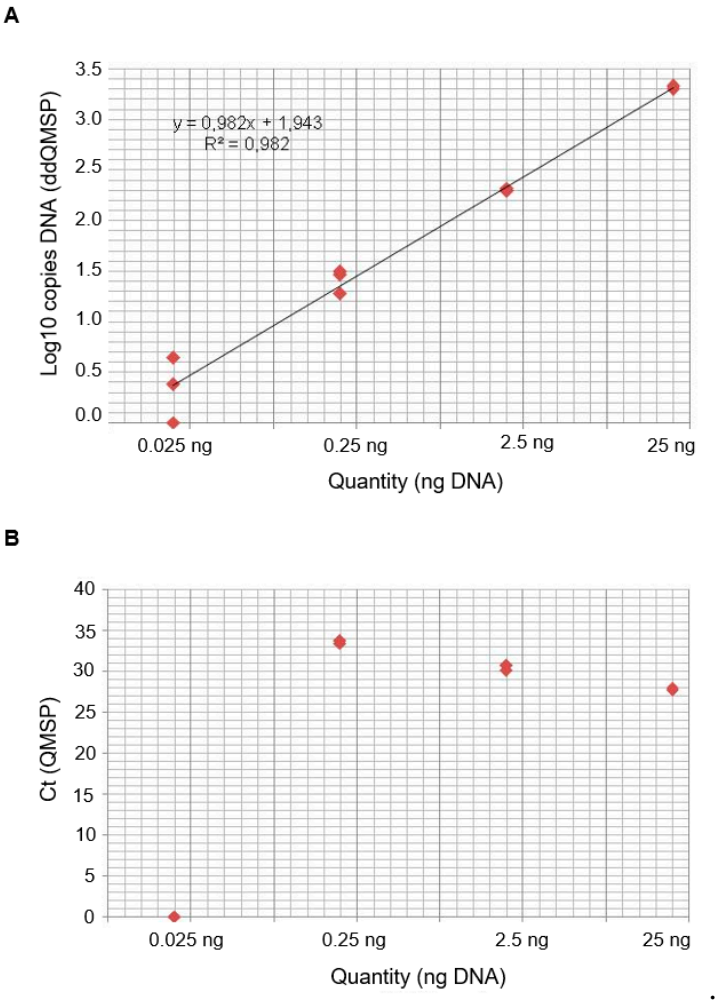


Figure S3: Comparative Linear Regression analysis of limit of detection (LOD) for *B4GALT1* promoter methylation between dd-QMSP and QMSP. For both assays 10-fold serial dilutions (250ng, 25ng, 2.5ng, 0.25ng, 0.025 ng) of 100% Methylated Control DNA were used to construct a calibration curve. (**A**) The log10-transformed 100% methylated DNA copy numbers determined by dd-QMSP were plotted against the corresponding standard curve dilutions (**B**) The ct values of 100% methylated DNA determined by QMSP were plotted against the corresponding standard curve dilutions.