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Reduced aquaporin-1 transcript expression in colorectal carcinoma is associated with promoter hypermethylation

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TITLE: Reduced aquaporin-1 transcript expression in colorectal carcinoma is associated with promoter hypermethylation

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ABSTRACT

Aquaporin-1 (AQP1) is a homo-tetrameric transmembrane protein that facilitates rapid movement of water and ions across cell membranes. The clinical significance of AQP1 expression in colorectal carcinoma (CRC) is controversial. The aim of this study was to investigate the prognostic significance of AQP1 transcript expression and the association between expression and promoter methylation in normal colonic mucosa, CRC tissues and cell lines. Analysis of publicly available datasets from The Cancer Genome Atlas revealed that AQP1 expression was significantly decreased in CRC compared to normal mucosa (12.7 versus 33.3 respectively, $P < 0.0001$). However, expression increased with advanced disease, being significantly higher in stage IV (17.6) compared to either stage I (11.8, $P = 0.0039$) or II (10.9; $P = 0.0023$), and in patients with lymph node metastasis compared to those without (13.9 versus 11.3 respectively, $P = 0.0023$). Elevated expression was associated with decreased overall survival with univariate (Cox Proportional Hazard Ratio 1.60, 95% confidence interval 1.05-2.42, $P = 0.028$), but not multivariable analysis when considering the confounders stage and age. Analysis of HumanMethylation450 data demonstrated that AQP1 promoter methylation was significantly increased in CRC compared to normal mucosa. Analysis of CRC tissues and cell lines strongly suggested that methylation was associated with decreased expression. *BRAF*^{V600E} mutation alone did not explain the increase in methylation. In conclusion, AQP1 transcript expression was decreased in CRC compared to normal mucosa, and this was associated with AQP1 promoter hypermethylation. AQP1 transcript expression increased with advanced disease but was not an independent prognostic indicator.

KEYWORDS

Colorectal cancer; Aquaporin-1 (AQP1); Expression; DNA methylation; Prognosis

INTRODUCTION

Globally, colorectal carcinoma (CRC) is one of the most commonly diagnosed cancers and is a leading cause of cancer-related deaths [1]. At diagnosis, up to 25% of patients present with metastatic disease (stage IV). Additionally, up to 25% of patients diagnosed with early localised disease (stage I or II) and 50% with locally advanced CRC (stage III) eventually relapse with overt metastatic disease following surgery with curative intent [2]. Despite recent advances in treatment, patients with metastatic disease currently have 5-year survival rates of less than 15%, and a median overall survival of approximately 30 months [3, 4]. There is a need to improve staging and treatment of CRC.

Aquaporin-1 (AQP1) forms a homo-tetrameric transmembrane channel that facilitates the rapid movement of water and ions across cell membranes in response to osmotic gradients [5]. Increased expression has been reported in numerous cancer types including brain, breast, cervical, lung, renal, and CRC [6]. Previous studies have suggested that AQP1 is involved in enhancing cancer cell migration and invasion (reviewed in[6]). Studies comparing AQP1-null and wildtype mice suggest that AQP1 promotes tumour growth by enhancing endothelial cell migration and angiogenesis [7]. Whilst the relevance of AQP1 expression has been investigated in numerous cancer types, only a small number of studies have investigated the prognostic significance of AQP1 protein expression in CRC, and these have yielded conflicting results [8, 9, 10].

Changes in DNA methylation are frequent, early events in carcinogenesis [11]. DNA methylation is of clinical interest because it might lead to biomarkers for early detection, diagnosis, prognostication, therapeutic stratification, and post-therapeutic monitoring.

Promoter methylation plays a role in mediating gene expression, as evidenced by studies showing that methylation of gene promoter regions varies considerably depending on the cell type, with more methylation correlating with low or no transcription [12]. This has been highlighted in cancer, as experimentally shown by some research groups, which show that hypermethylation occurs in cancer at promoters of genes already transcriptionally repressed [13, 14]. However, studies on the association between AQP1 promoter methylation and gene expression are currently limited to glioblastoma multiform, normal salivary gland and salivary gland adenoid cystic carcinoma [15, 16, 17], and have not been reported for other carcinomas including CRC.

The aim of this study was to investigate the prognostic significance of AQP1 transcript expression and the association between expression and promoter methylation in colon cancer cell lines, normal colonic mucosa and CRC tissues.

RESULTS

Expression of AQP1 was reduced in colorectal carcinoma compared to normal mucosa

Expression of AQP1 transcript was analysed in the combined The Cancer Genome Atlas (TCGA) colon (TCGA-COAD) and rectal adenocarcinoma (TCGA-READ) datasets. Transcript expression data, reported as the median number of fragments per kilobase of exon per million reads (FPKM), were available for 590 patients with confirmed CRC, 47 of which had matched normal colonic mucosa (summary of clinicopathological features in Table S1). There was no significant difference in the median age of the 590 CRC patients (68 years, range 31-90) compared to those with normal mucosa (73 years, range 40-90; $P = 0.0585$). Expression of AQP1 was significantly higher in normal mucosa (median 33.3, range 11.2-146.4) compared to either all CRC (median 12.7, range 0.8-175.9, $P < 0.0001$) or to patient

matched CRC (median 15.8, range 3.9-126.7, $P < 0.0001$). Expression was higher in the normal mucosa compared to CRC for 40 of 47 patients (85 %). Analysis of publicly available datasets deposited in Oncomine (www.oncomine.org) provided further supportive evidence that AQP1 transcript expression was significantly higher in normal mucosa compared to either CRC or adenoma (Table S2). Together, these data strongly suggest that AQP1 transcript expression was significantly decreased in CRC compared to normal mucosa.

Analysis of the TCGA datasets by disease stage revealed that expression was significantly higher in normal mucosa compared to either stage I (median 11.8, range 0.8-138.5, $P < 0.0001$), II (median 10.9, range 2.0-126.7, $P < 0.0001$), III (median 13.9, range 2.2-175.9, $P < 0.0001$), or IV (median 17.6, range 2.0-153.7, $P = 0.0003$) CRC (Table 1). Expression increased with advanced stage, being significantly higher in stage IV compared to either stage I ($P = 0.0039$) or II ($P = 0.0023$), and in patients with lymph node metastasis (stage III) compared to those without (stage I-II, median 13.9, range 2.2-175.9, $P = 0.0039$). Expression was significantly less in right-sided compared to left-sided CRC (median 11.4, range 0.8-175.9 versus median 13.4, range 2.0-153.7 respectively, $P = 0.0284$). It was not significantly different for gender, age, morphology, or *BRAF* mutation status.

Reduced AQP1 expression was associated with increased overall survival in univariate but not multivariable analysis

Kaplan-Meier analysis of the overall survival (OS) for the 590 patients represented in the combined TCGA datasets demonstrated that elevated AQP1 expression (defined as a FPKM ≥ 11.4) was associated with significantly poorer OS ($P = 0.0142$; Figure 1). A subset analysis was performed on 453 of the 590 patients with complete clinical data. Elevated AQP1 transcript expression was associated with poorer OS in the univariate (Table 2; hazard ratio

(HR) 1.60, 95% confidence interval (CI) 1.05-2.42, $P = 0.028$) but not the multivariable model (HR 1.15, 95% CI 0.75-1.77, $P = 0.523$). Age and stage of cancer remained significant, independent predictors of OS in the multivariable model: in summary, each 1 year increase in age was associated with a 4% increase in risk of dying; when compared to patients with stage I-II cancer, the risk of dying was 2.6 times higher amongst patients with stage III and 7.66 times higher amongst those with stage IV cancer.

AQP1 promoter was frequently hypermethylated in CRC compared to normal mucosa

Having identified that AQP1 transcript expression was decreased in CRC compared to normal mucosa, we examined if the reduced expression was associated with AQP1 DNA methylation. Infinium HumanMethylation450 BeadChip (HM450) DNA methylation data were available for 317 of the 590 CRC patients (225 from TCGA-COAD and 92 from TCGA-READ), 32 of which had patient matched normal mucosa. Methylation data, expressed as beta-values, for AQP1 were available for 21 CpG probesets, of which 10 were located -5,000 to -1 bp upstream of the transcription start site (TSS), eight were in the gene body defined as +1 to +13,824 bp downstream of the TSS, and two were in the distal intergenic region within 10,000 bp of the end of the gene body. There were no overt differences in methylation of these probesets between colon and rectal carcinoma. Statistically significant differences in average beta-values between normal mucosa and CRC were observed for 16 of the 21 probesets (Figure 2, Figure S1 and Figure S2; Bonferroni adjusted $P < 0.05$). For the eight probesets located between -200 and +200 bp of the TSS the average beta-values for normal mucosa were < 0.2 and were significantly less than the average beta-values for CRC (Figure 2). Methylation was significantly higher (hypermethylated) in CRC compared to normal mucosa for all 13 probesets in the region -385 to +1,653 bp of the TSS and for the probeset at -658 bp (Figure 2 and Figure S2;

Bonferroni adjusted $P < 0.05$). In contrast, for the 2 probesets located in the distal intergenic region, at +13,977 and +21,814 bp, methylation was significantly lower in CRC compared to normal mucosa. No significant differences were observed for the probesets at -4,832, -407, +3,051, +9,315 and +12,504 bp. Differential methylation (deltaBeta, defined as a difference in average beta-values for normal mucosa subtracted from CRC of > 0.2) was observed for 6 of 18 probesets (33.3 %) located within region from -658 bp to the end of the gene body (Figure 2 and Figure S1). This data clearly demonstrates that the AQP1 promoter was hypermethylated in CRC compared to normal mucosa.

Reduced AQP1 expression correlated with promoter methylation in colorectal carcinoma tissues and colon cancer cell lines

We investigated if AQP1 promoter methylation was associated with decreased expression in CRC tissues and cell lines. Expression and methylation data were available for the 317 CRC samples from the combined TCGA datasets. Statistically significant inverse correlations were observed for 15 of 16 probesets located in the region -658 to +3,051 bp from the TSS (Figure S3, Bonferroni adjusted $P < 0.05$). When the CRC samples were stratified based on low (FPKM < 11.4) or high (FPKM ≥ 11.4) AQP1 expression, low expression was associated with significant increases in methylation for 8 of 9 probesets located in the region -90 to +3,051 bp of the TSS and for the probeset at -658 bp (Figure 3 and Figure S4; Bonferroni adjusted $P < 0.05$). This data suggest that AQP1 promoter methylation was associated with reduced transcript expression in CRC tissues.

Next, we examined if reduced expression of AQP1 was associated with promoter methylation in colon cancer cell lines. Analysis of microarray gene expression datasets for cell lines that constitute the National Cancer Institute's NCI60 panel, representing breast, central nervous

system, colon, leukemia, melanoma, non-small cell lung cancer, ovarian, prostate and renal tumours, revealed that typically the highest expression of AQP1 was observed in the colon cancer cell line COLO 205. Of the seven colon cancer cell lines represented in the NCI60 panel, expression was relatively high in COLO 205, moderate in HT-29 and HCC2998 and low in SW620, KM12, HCT 116 and HCT 15 (Figure 4a). We independently validated these findings in COLO 205, HT-29 and HCT 116 using AQP1 TaqMan Gene Expression Assays (Figure 4b). Expression of AQP1 was significantly higher in COLO 205 compared to HT-29 ($P < 0.0001$), and in HT-29 compared to HCT 116 ($P < 0.0001$).

Analysis of the DNA methylation for the colon cancer cell lines stratified based on the level of AQP1 transcript expression suggested that low expression was associated with an increase in methylation for the probesets located near the TSS (Figure 4c and Figure S5; $P < 0.05$). Differences were observed for 5 of 10 probesets located within the promoter region between -5,000 and -1 bp upstream of the TSS, and in 1 of 9 probesets in the gene body. Differential methylation (deltaBeta > 0.2) calculated by subtracting the average beta-value of the high to moderate from the low expressing cell lines was observed in 13 of 21 probesets (Figure 4c); all 10 probesets in the region from -407 to +27 bp and the three probesets in the region from +1653 to +9,315 bp.

Next, we analysed the transcriptional response to the global demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC). Analysis of a publicly available dataset [18] demonstrated that treatment of HT-29 with 5 and 10 μM 5-aza-dC for 5 days induced significant upregulation of AQP1 transcript expression (Figure 4d; $P = 0.0004$ and $P = 0.0171$, respectively). To confirm and expand on these findings, we measured changes in AQP1 expression in COLO 205, HT-29, and HCT 116, which display low, intermediate and high AQP1 promoter

194 methylation respectively (Figure S5), treated with 5-aza-dC (Figure 4e). Treatment of COLO
195 205 with 1 µM resulted in a slight (1.4-fold), statistically significant increase in AQP1
196 expression ($P = 0.0031$). In contrast, treatment of HT-29 and HCT 116 with either 1, 5 or 10
197 µM aza-dC resulted in marked (> 3.6-fold for all), significant increases in AQP1 expression
198 ($P < 0.0001$ for all), with the magnitude of the changes being greater in HCT 116. Together,
199 these data provide strong evidence that reduced AQP1 transcript expression was associated
200 with promoter hypermethylation in CRC tissues and colon cancer cell lines.
201
202 ***The BRAF^{V600E} mutation alone was not associated with AQP1 promoter hypermethylation***
203 The mechanisms driving aberrant AQP1 promoter hypermethylation that was observed in
204 CRC are unknown. The *BRAF*^{V600E} mutation is known to be associated with high CpG island
205 methylator phenotype (H-CIMP) in CRC. We investigated if the *BRAF*^{V600E} mutation was
206 associated with AQP1 methylation in tissues and cell lines. Of the 317 CRC with methylation
207 data, 275 had wildtype *BRAF*, 26 had a *BRAF*^{V600E} mutation, and 16 had other *BRAF*
208 mutations. Differential methylation (deltaBeta > 0.2) between wildtype and *BRAF*^{V600E} CRC
209 was not observed for any of the probesets (Figure 5a). Methylation was significantly greater
210 in CRC that harboured the *BRAF*^{V600E} mutation compared to wildtype for 1 of 21 probesets,
211 located +1,653 bp from the AQP1 TSS (Figure S6; Bonferroni adjusted $P < 0.05$).
212
213 Finally, we investigated if the *BRAF*^{V600E} mutation was associated with AQP1 promoter
214 methylation in the NCI60 cell lines. Analysis of the mutation data deposited in the Catalogue
215 of Somatic Mutation in Cancer database (COSMIC; http://cancer.sanger.ac.uk/cosmic, [19])
216 demonstrated that the *BRAF*^{V600E} mutation was present in two of seven colon cancer (COLO
217 205 and HT-29) and seven of nine melanoma cell lines. Another two cell lines, the colon
218 cancer line KM12 and the breast cancer line MDA-MB-231, had other mutations in *BRAF*.

One of the 60 cell lines (NCI-ADR-RES) was excluded from the analysis because it was known to be contaminated with OVCAR-8. In contrast to CRC tissues, reduced differential methylation (deltaBeta < -0.2) was observed in the cell lines with the *BRAF*^{V600E} mutation compared to wildtype for 3 of 21 probesets, located at -385, -90, and -39 bp from the TSS (Figure 5b). However, differential methylation was not statistically significant for any of the 21 probesets analysed (Figure S7).

DISCUSSION

Our analysis of publicly available datasets revealed that AQP1 transcript expression was significantly decreased in CRC compared to normal mucosa. In CRC, expression increased with advanced disease, and was associated with a significant decrease in OS with univariate but not with multivariable analysis when the confounders stage and age were considered. AQP1 promoter methylation was significantly increased in CRC compared to normal mucosa, and analysis of CRC tissues and cell lines strongly suggested that AQP1 promoter hypermethylation was associated with decreased expression. The *BRAF*^{V600E} mutation alone did not explain the increase in promoter methylation.

We report the novel findings that the AQP1 promoter was hypomethylated in normal colonic mucosa, hypermethylated in a significant proportion of CRC, and methylation was associated with decreased transcript expression in CRC patient tissues and colon cancer cell lines. Further, treatment of colon cancer cell lines with a global demethylating agent, 5-aza-dC, significantly increased AQP1 expression. To the best of our knowledge, regulation of AQP1 expression by promoter methylation has previously only been demonstrated in glioblastoma multiform, normal salivary tissue and an adenoid cystic carcinoma cell line (SACC83), and a hypomethylated promoter was associated with a relative increase in

expression in a proportion of salivary gland adenoid cystic carcinomas [15, 16, 17]. Treatment of the SACC83 cell line with global demethylating agents, 5-aza-dC and trichostatin A, decreased AQP1 promoter methylation and upregulated expression, providing further supportive evidence that AQP1 promoter methylation was associated with a reduction in transcript expression [16].

There are limited published data comparing AQP1 expression in normal colonic mucosa to CRC. Imaizumi et al reported that AQP1 protein was not detected in the normal epithelial cells but was expressed in 112 of 268 (41.8 %) of stage 0 to IV CRC [10]. Mobasheri et al evaluated the relative abundance and distribution of AQP1 protein using immunohistochemistry and quantitative histomorphometric analysis of tissue microarrays. They reported that AQP1 was expressed in capillary endothelia of normal tissues, and expression was higher in the microvascular structures of CRC. AQP1 was observed in some neoplastic tumour cells, however they did not comment on expression in normal colonic mucosa [20]. Moon et al using *in situ* hybridization reported strong AQP1 transcript expression in the colon cancers and adenomas from 12 patients, with almost no expression in adjacent normal mucosa [21]. In contrast, we had previously reported using TaqMan PCR that AQP1 transcript expression was higher in patient matched normal colonic mucosa compared to CRC for 35 of 57 (61 %) patients [22]. Confirming our previous findings, we report here that AQP1 transcript expression was significantly greater in normal mucosa compared to CRC for 40 of 47 (85 %) patient matched samples available in combined TCGA datasets. Furthermore, analysis of multiple publicly available datasets in Oncomine provided further supportive evidence that transcript expression was significantly greater in normal mucosa compared to CRC. However, a limitation of these studies that assessed AQP1

transcript expression is that we cannot be certain that transcript expression was restricted to the epithelia, nor that transcript levels translated to protein expression.

Previous reports found that AQP1 protein was expressed in 40-60% of CRC. Yoshida et al reported expression in 56 of 120 (46.7%) patients with stage II and III colon cancer [8]. Imaizumi et al reported expression in 112 of 268 (41.8%) patients with stage 0-IV CRC [10]. In the largest study previously reported, Kang et al reported strong positive staining in 298 of 486 patients with stage I-III colon cancer (61.3%) [9]. Our finding that AQP1 promoter methylation was associated with decreased transcript expression provides an explanation for the lack of expression in a significant proportion of CRC.

The clinical significance of AQP1 protein expression in CRC has yielded conflicting results. Yoshida et al reported that expression was associated with decreased OS and multivariable analysis suggested that AQP1 expression was an independent prognostic risk factor (Risk Ratio 2.593, 95% CI 1.057-6.439, $P = 0.038$) [8]. The multivariable model consisted of the confounders age ($\geq$ 75 years), site of primary, presence of bowel obstruction, depth of invasion, lymph node involvement, and elevated carcinoembryonic antigen. Expression was more common in left compared to right-sided tumours and was associated with lymph node involvement, lymphovascular and vascular invasion. More recently, Imaizumi et al reported that expression was associated with increased depth of invasion, lymph node metastasis, lymphatic invasion, and venous invasion [10]. Positive expression was more common in left compared to right-sided tumours, and in moderately compared to well differentiated tumours. They did not report on OS. Expression was not associated with disease-free survival (DFS) in patients with stage II and III CRC following surgery with curative intent. Interestingly, amongst the 84 patients with stage II and III disease that received adjuvant chemotherapy,

low AQP1 expression was associated with decreased DFS (HR 0.45, 95% CI 0.21-1.00, *P* = 0.05). In contrast to the other studies, Kang et el reported that strong positivity was associated with decreased lymph node metastasis [9]. They did not report on expression by side. Expression did not correlate with either OS or DFS.

The reasons for the conflicting results between previous studies assessing protein expression are uncertain. It may be due to the lack of standardisation between studies. The immunohistochemical staining protocol varied between studies. Each study used a different anti-AQP1 antibody and it was not clear if the antibodies had been validated. Different antigen retrieval protocols were used. The definition of AQP1-positivity and the scoring protocol varied between studies. Membranous and cytoplasmic staining of tumour cells was assessed in two studies [9, 10], but it was not defined in the other study [8]. These differences make direct comparisons between the studies and interpretation of the results difficult.

Previous studies suggest that AQP1 promotes tumour progression by enhancing cancer cell migration and metastasis, and by increasing endothelial migration and microvessel density (reviewed in [6]). *In vitro* studies have demonstrated that knockdown of AQP1 reduced viability, migration and invasion and promoted apoptosis in ovarian cancer cells [23], induced apoptosis and inhibited proliferation, adhesion and invasion in osteosarcoma cells [24], significantly decreased migration and invasion of lung cancer cells [25], reduced the migration capacity of melanoma [26], colon cancer [27], and endothelial cells [26]. Overexpression of AQP1 has been shown to accelerate cell migration *in vitro* [7, 27]. *In vivo* studies have demonstrated that overexpression of AQP1 in colon cancer cells increased pulmonary extravasation in a metastatic murine model [27]. AQP1 knockdown by intratumoural injection of siRNA reduced tumour volume and microvessel density in a

murine melanoma model [28]. Furthermore, AQP1 knockout mice developed smaller tumours with reduced microvessel density and fewer metastases compared to wildtype mice [7, 29].

The mechanisms leading to AQP1 promoter methylation in CRC are currently unclear. Mutations in *BRAF* are known to alter its kinase activity and subsequently impact on the activation of mitogen-activated protein pathway [30]. In melanoma, $BRAF^{V600E}$ mutation was associated with an increase in AQP1 expression, and increased expression was associated with decreased progression-free and overall survival [31]. In CRC, $BRAF^{V600E}$ mutation was associated with a high CpG island methylator phenotype (H-CIMP) [32, 33]. However, we did not find a consistent association between $BRAF^{V600E}$ mutation and AQP1 promoter hypermethylation in CRC tissues and colon cancer cell lines. In CRC tissues, methylation of only one probeset, located at +1653 bp from the TSS, was significantly higher in tumours that harboured a $BRAF^{V600E}$ mutation compared to wildtype. In contrast, $BRAF^{V600E}$ colon cancer cell lines typically had lower AQP1 promoter methylation and expressed higher levels of AQP1 transcript compared to wildtype. Together, this suggests that a $BRAF^{V600E}$ mutation alone does not explain aberrant AQP1 promoter methylation.

In conclusion, AQP1 transcript expression was significantly decreased in CRC compared to normal colonic mucosa. Reduced expression was associated with AQP1 promoter hypermethylation and an increase in overall survival with univariate but not multivariable analysis. The $BRAF^{V600E}$ mutation did not appear to explain the aberrant AQP1 promoter methylation.

MATERIALS AND METHODS

In silico data

For tissues, RNA sequencing expression (median number of fragments per kilobase of exon per million reads, FPKM), Infinium HumanMethylation450 BeadChip (HM450) DNA methylation (beta-values), and *BRAF* mutation data were obtained from TCGA Research Network (http://cancergenome.nih.gov/). Expression data were available for a total of 597 CRC patients deposited in the combined TCGA-COAD (n = 438) and TCGA-READ (n = 159) datasets. Of these, seven samples were excluded because the site of primary diagnosis could not be confirmed as CRC; for four samples the site was not recorded, two were coded as malignant neoplasm of the connective and soft tissue of abdomen, and one was coded as malignant (primary) neoplasm, unspecified. A total of 590 CRC patients had AQP1 transcript expression data for subsequent analyses (Additional file 1: Table S1).

For the NCI60 cancer cell lines, microarray gene expression were downloaded from Gene Expression Omnibus (GEO) Datasets using accession numbers GSE32474 [34] and GSE2003 [35]. HM450 data for the NCI60 were downloaded using GEO Datasets accession number GSE49143. BRAF mutation data were obtained COSMIC (http://cancer.sanger.ac.uk/cosmic) [19].

For the RNA-Seq transcriptomic profiles generated from 5-aza-deoxycytidine treated HT-29 colon cancer cells, data were downloaded from GEO using the accession number GSE41586 [18].

Cell lines

Colon cancer cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cell lines were maintained in either RPMI-1640 (COLO 205) or

DMEM (HT-29 and HCT 116) supplemented with 10% foetal bovine serum, 1x Glutamax, 200 U/mL penicillin, and 200 mg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37^{o}C in a humidified incubator with 5% CO_2 in air.

Treatment with 5-aza-2'-deoxycytidine

To study the effects of the global demethylating agent 5-aza-2'-deoxycytidine (5-aza-dC), 2.5 x 10^4 cells were seeded into triplicate wells of a 6-well plate. The following day, the media were replaced with fresh media supplemented with either 0, 1, 5 or 10 µM 5-aza-dC (Sigma-Aldrich, St Louis, MO, USA), as described previously [36, 37, 38], and the cells were treated for five days.

Analysis of AQP1 expression by quantitative PCR

Total RNA was isolated using the PureLink RNA Mini Kit (Thermo Fisher Scientific), and 200 ng was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) in a final volume of 20 µL. Transcript expression was determined using duplex TaqMan Gene Expression Assays for AQP1 (Hs01028916_m1) and phosphomannose mutase 1 (PMM1; Hs00963625_m1; Thermo Fisher Scientific) using Sso Advanced Universal Probes Supermix (Bio-Rad Laboratories) as described previously [39], using a ViiA7 Real-Time PCR System (Thermo Fisher Scientific). Results were calculated using the comparative CT method for relative quantitation ($2^{-\Delta\Delta CT}$).

Statistical analysis

Patients were stratified into high or low AQP1 transcript expression using the best separation of the combined TCGA RNA sequencing expression data. To choose the best FPKM cut-off for grouping the patients most significantly, all FPKM values from the 20th to 80th

percentiles were used to group the patients, significant differences in the survival outcomes of the groups were examined by Kaplan-Meier survival estimators and the value yielding the lowest log-rank *P* value was selected. The prognosis of each group of patients was examined, and the survival outcomes of the two groups were compared by the log-rank test using Prism v7.0d for Mac OS X (GraphPad Software, Inc. La Jolla, CA, USA).

The association between AQP1 expression and overall survival was assessed using Cox Proportional Hazards models. Unadjusted hazard ratios are reported along with the hazard ratio adjusted for other significant and independent predictors of survival (age and stage). The analysis included all 453 patients with complete data on the outcome and predictor variables. All tests were two-tailed and assessed at the 5% alpha level. The analyses were conducted using SAS v9.4 (SAS Institute Inc., Cary, NC, USA).

Comparisons of methylation (beta-values) between groups for individual probesets were assessed using unpaired Welch's t-test. Correlations between AQP1 expression and methylation in CRC were assessed using the Spearman correlation coefficient. *P* values were adjusted for multiple comparisons using the Bonferroni correction. Comparison between relative quantitation of AQP1 transcript expression between cell lines and in response to 5-aza-dC treatment was determined using unpaired t-test and one-way analysis of variance with Dunnett's multiple comparisons test. All tests were two-tailed and assessed at the 5% alpha level. The analyses were conducted using Prism v7.0d for Mac OS X.

LIST OF ABBREVIATIONS

AQP1: aquaporin-1; **bp:** base pairs; **COSMIC:** Catalogue of Somatic Mutation in Cancer database; **CRC:** colorectal carcinoma; **DFS:** disease-free survival; **FPKM:** fragments per

kilobase of exon per million reads; **GEO:** Gene Expression Omnibus; **H-CIMP:** high CpG island methylator phenotype; **HM450:** Infinium HumanMethylation450 BeadChip; **HR:** hazard ratio; **IQR:** interquartile range; **NCI60:** National Cancer Institute's 60 human tumour cell lines; **NOS:** not otherwise specified; **NR:** not recorded; **ns:** not significant; **OS:** overall survival; **TCGA:** The Cancer Genome Atlas; **TCGA-COAD:** The Cancer Genome Atlas colorectal adenocarcinoma; **TCGA-READ:** The Cancer Genome Atlas rectal adenocarcinoma; **TSS:** transcription start site.

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

For tissues, RNAseq expression, HM450 DNA methylation, and *BRAF* mutation data were obtained from TCGA Research Network (http://cancergenome.nih.gov/). For the NCI60 cell lines, microarray gene expression and HM450 DNA methylation data were downloaded from GEO Datasets using accession numbers GSE32474, GSE2003, and GSE49143. *BRAF* mutation data for the cell lines was obtained COSMIC. For the HT-29 colon cancer cells treated with 5-aza-dC, RNA-Seq data were downloaded from GEO Datasets using the accession number GSE41586.

Competing interests

The authors declare they have no competing interests.

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Authors' contributions

ES and JEH conceived the paper. ES designed and performed the analysis for all the experiments, except SH performed univariate and multivariable Cox Proportional Hazards models. ES prepared the manuscript. All authors read, revised and approved the final manuscript.

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FIGURE LEGENDS

Figure 1: High expression of AQP1 was associated with shorter overall survival in colorectal carcinoma. RNA sequencing data reported as median number of fragments per kilobase of exon per million reads (FPKM) and survival data for the 590 patients in the combined TCGA-COAD and TCGA-READ datasets were obtained from TCGA Research Network (http://cancergenome.nih.gov/). Low AQP1 transcript expression (n = 261) was defined as FPKM < 11.4, and high (n = 329) as FPKM ≥ 11.4.

Figure 2: Methylation of AQP1 in normal mucosa and colorectal carcinoma from patients in the combined TCGA-COAD and TCGA-READ datasets. Average beta-values for all available individual probesets located in the region -500 to +500 bp from the AQP1 transcription start site (TSS) for normal mucosa (N, n= 32) and colorectal carcinoma (CRC, n = 317). Differential methylation (deltaBeta) was calculated by subtracting the average beta-value of N from CRC. Comparisons between N and CRC were considered statistically significant when the adjusted *P* value (adj. *P*) for the unpaired Welch's t-test with Bonferroni correction for multiple comparisons was < 0.05. * adj. $P < 0.05$.

Figure 3: Methylation of AQP1 in low and high AQP1 expressing colorectal carcinoma from patients in the combined TCGA-COAD and TCGA-READ datasets. Average beta-values for all available individual probesets located in the region -500 to +500 bp from the AQP1 transcription start site (TSS) for AQP1 low (n = 127) and high (n = 190) expressing colorectal carcinoma. Differential methylation (deltaBeta) was calculated by subtracting the average beta-value of AQP1 high from AQP1 low expressing CRC. Comparisons between AQP1 low and AQP1 high were considered statistically significant when the adjusted *P* value (adj. *P*) for the unpaired Welch's t-test with Bonferroni correction for multiple comparisons was < 0.05. * adj. $P < 0.05$.

Figure 4: Low AQP1 expression was associated with increased promoter methylation in the NCI60 colon cancer cell lines. a) AQP1 transcript expression in colon cancer cell lines from the NCI60 panel. Microarray gene expression data for the NCI60 panel were downloaded from Gene Expression Omnibus Datasets (GEO) using accession numbers GSE32474 and GSE2003, and log2 data were expressed relative to the expression in COLO 205. Data for GSE32474 are the average of triplicate microarrays. b) Relative quantitation of

AQP1 transcript expression was determined by the comparative CT method ($2^{-\Delta\Delta CT}$) in colon cancer cell lines using duplex TaqMan Gene Expression Assays, with PMM1 as endogenous control. Data are relative to COLO 205. Each data point is the average of triplicate reactions for three biological replicates, with bars representing the mean with standard deviation. c) AQP1 methylation in colon cancer cell lines from the NCI60 panel. Beta-values were downloaded using GEO accession number GSE49143. Average beta-values were calculated for the low (HCT 15, KM12, SW620 and HCT 116) and the high to moderate (high/mod.) AQP1 expressing colon cancer cell lines (COLO 205, HT-29, HCC2998). Differential methylation (deltaBeta) was calculated by subtracting the average beta-value of the high/mod. from the low expressing cell lines. Comparisons between low and high/mod. were considered statistically significant when the *P* value for the unpaired Welch's t-test was < 0.05. * $P < 0.05$. d) RNA-Seq data for HT-29 treated with 0, 5 or 10 µM 5-aza-dC for five days were downloaded using accession number GSE41586. Data are the mean with standard deviation of three biological replicates, expressed relative 0 µM 5-aza-dC. e) Relative quantitation of AQP1 transcript expression in COLO 205, HT-29 and HCT 116 following five days of treatment with 5-aza-dC. Data are the mean with standard deviation of three biological replicates, expressed relative 0 µM 5-aza-dC.

Figure 5: AQP1 methylation in colorectal carcinoma tissues and cell lines according to *BRAF*^{V600E} mutation status. Average beta-values for all individual probesets located in the region from -5,000 to +25,000 bp of the AQP1 transcription start site (TSS) for a) TCGA CRC tissues, and b) the NCI60 panel of cell lines. Differential methylation (deltaBeta) was calculated by subtracting the average beta-value of samples with wildtype from samples with a *BRAF*^{V600E} mutation.

659 **TABLES**

660 **Table 1. AQP1 transcript expression in tissues from the combined TCGA-COAD and**

661 **TCGA-READ datasets by clinicopathological characteristics**

	Number	**Median**	**Range**	**IQR**	***P* value**
Normal mucosa	47	33.3	11.2-146.4	21.4-48.9	
All CRC	590	12.7	0.8-175.9	7.9-23.8	< 0.0001
Gender					
Female	273	12.7	2-175.9	8.6-23.5	
Male	317	12.6	0.8-153.7	7.3-24.5	0.4444
Age, years					
< 50	71	12.7	2.0-138.5	9.0-24.7	
≥ 50 ≤ 65	189	14.7	0.8-175.9	8.8-28.2	
> 65	328	11.6	0.9-153.7	7.2-21.4	0.2704
NR	2	27.7	2.8-52.6	2.8-52.6	
Stage[1]					
I	102	11.8	0.8-138.5	7.6-17.4	0.0039
II	211	10.9	2.0-126.7	6.7-21.3	0.0023
III	172	13.9	2.2-175.9	8.5-25.2	0.7994
IV	85	17.6	2.0-153.7	9.9-29.7	
NR	20	16.9	5.8-53.2	8.6-28.3	
Lymph node metastasis					
No (stage I-II)	313	11.3	0.8-138.5	7.0-19.7	
Yes (stage III)	172	13.9	2.2-175.9	8.5-25.2	0.0039
Site of primary[2]					
Right	209	11.5	0.8-175.9	7.2-19.9	

Left	283	13.4	2.0-153.7	8.5-25.4	0.0284
Large intestine, NOS	98	14.7	2.2-138.5	8.3-22.2	
Histological subtype					
Adenocarcinoma, NOS	495	12.8	0.8-175.9	7.9-24.7	
Mucinous adenocarcinoma	75	12.2	2.0-112.0	9.0-22.0	0.9379
Other	20	10.2	4.2-129.1	7.7-23.3	
BRAF status					
Wildtype	526	12.6	0.8-175.9	7.9-24.1	
BRAF^{V600E} mutation	46	13.9	2.8-112.0	8.1-20.5	0.9341
Any *BRAF* mutation	64	13.9	2.8-112.0	8.1-21.9	0.8609

[1]Comparison to stage IV.

[2]Right-sided CRC was defined as caecum, ascending, hepatic flexure and transverse colon and left-sided as splenic flexure, descending, sigmoid, rectosigmoid and rectum.

NR, not recorded

NOS, not otherwise specified

Table 2. Overall survival for the 453 patients with complete clinical data in the combined TCGA-COAD and TCGA-READ datasets

	Univariate		**Multivariable**	
	HR (95% CI)	***P* value**	**HR (95% CI)**	***P* value**
AQP1 expression				
Low	1.00		1.00	
High	1.60 (1.05-2.42)	0.028	1.15 (0.75-1.77)	0.523
Age	1.03 (1.01-1.04)	0.003	1.04 (1.02-1.06)	< 0.0001
Stage				

I-II	1.00		1.00	
III	2.24 (1.35-3.71)	0.002	2.60 (1.56-4.34)	0.0002
IV	6.19 (3.79-10.25)	< 0.0001	7.66 (4.49-13.09)	< 0.0001
Gender				
Female	1.00			
Male	1.14 (0.77-1.71)	0.511		
Site of primary				
Left	1.00			
Right	1.32 (0.88-1.97)	0.175		
Histological subtype				
Adenocarcinoma, NOS	1.00			
Mucinous adenocarcinoma	1.23 (0.70-2.18)	0.473		
BRAF status				
Wildtype	1.00			
Any *BRAF* mutation	0.91 (0.47-1.76)	0.783		

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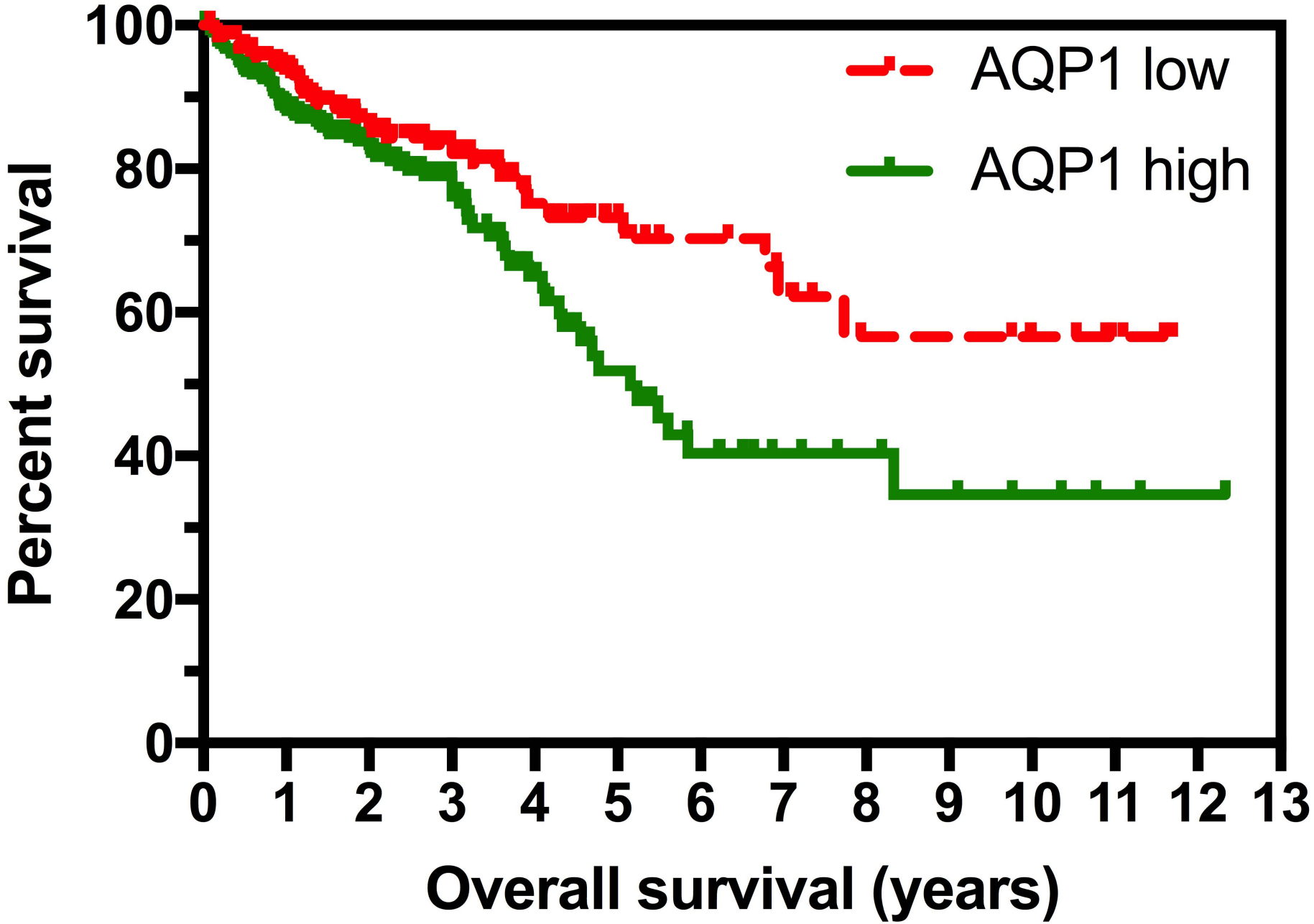
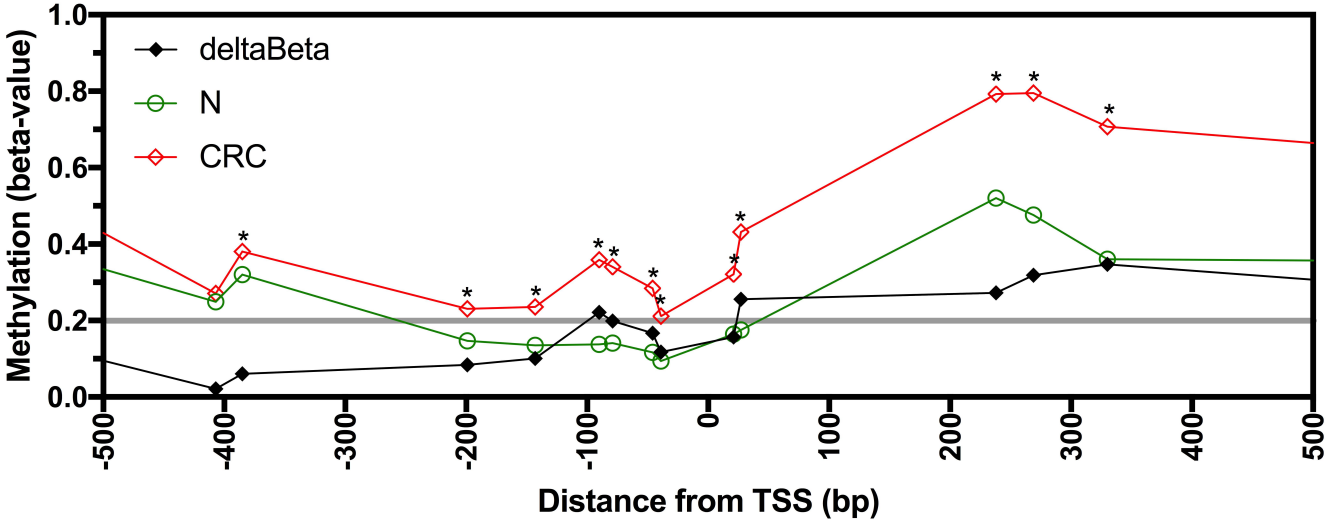
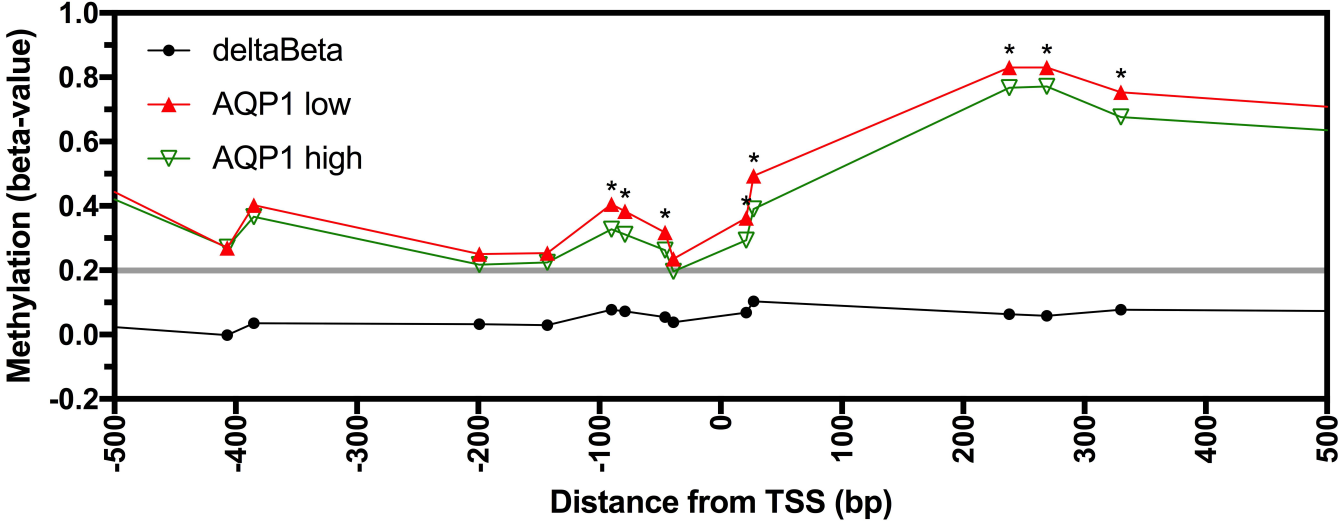
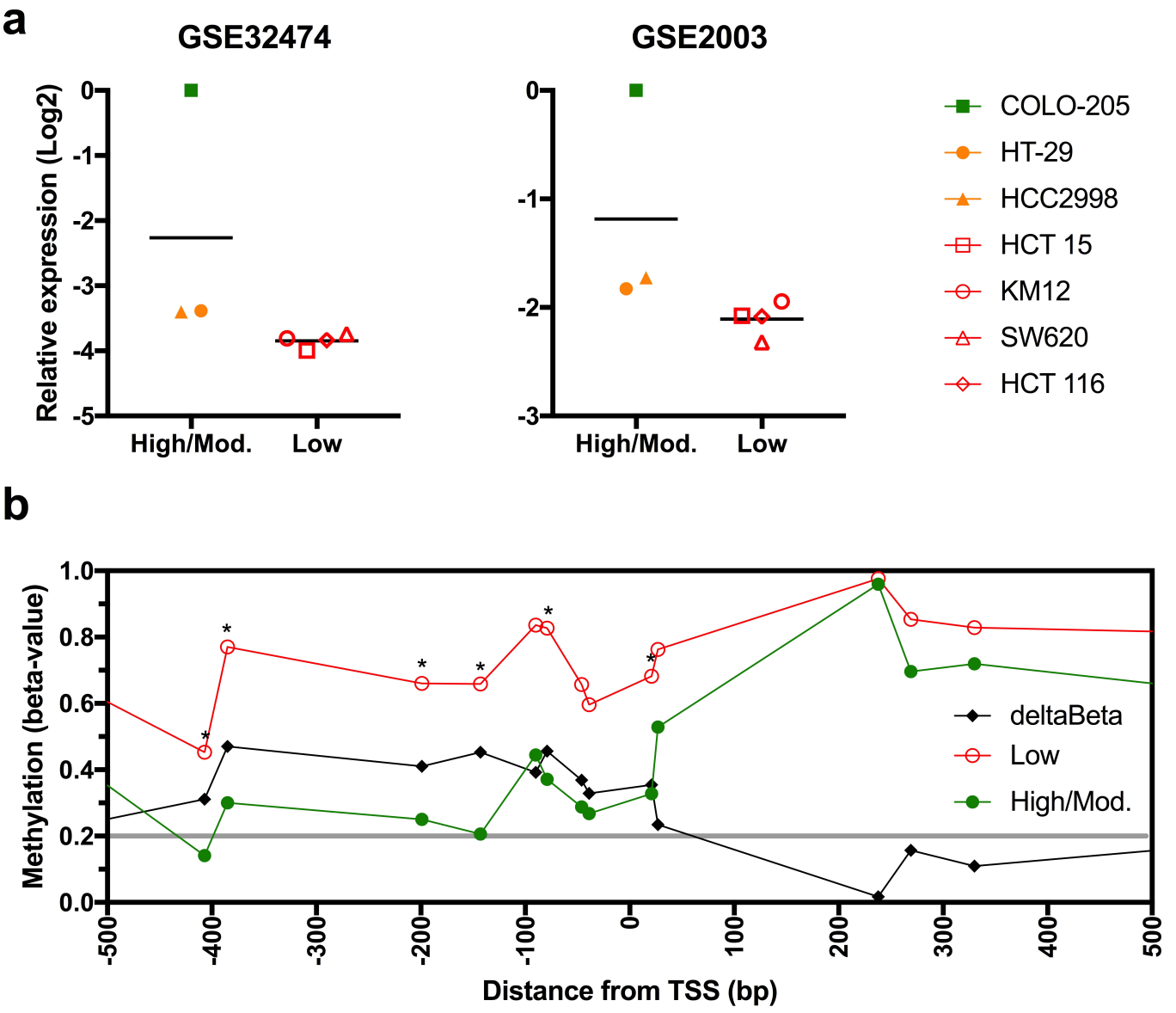
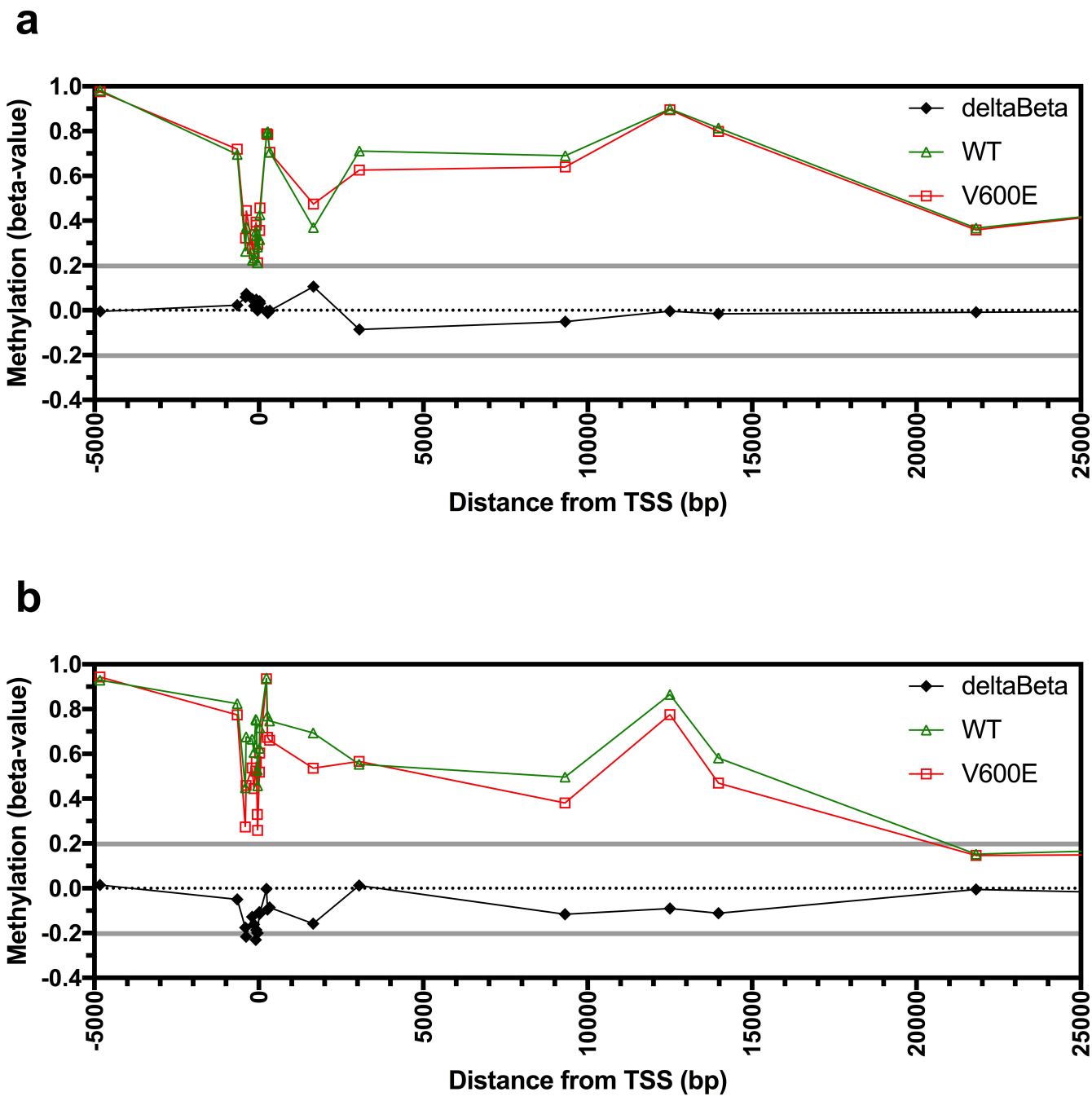

AQP1 low
AQP1 high
Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Overall survival (years)


deltaBeta
N
CRC
Methylation (beta-value)
1.0
0.8
0.6
0.4
0.2
0.0
-500
-400
-300
-200
-100
0
100
200
300
400
500
Distance from TSS (bp)


deltaBeta
AQP1 low
AQP1 high
Methylation (beta-value)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-500
-400
-300
-200
-100
0
100
200
300
400
500
Distance from TSS (bp)


a
GSE32474
GSE2003
Relative expression (Log2)
0
-1
-2
-3
-4
-5
High/Mod.
Low
COLO-205
HT-29
HCC2998
HCT 15
KM12
SW620
HCT 116
b
Methylation (beta-value)
1.0
0.8
0.6
0.4
0.2
0.0
-500
-400
-300
-200
-100
0
100
200
300
400
500
Distance from TSS (bp)
deltaBeta
Low
High/Mod.


a
b
Methylation (beta-value)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-5000
0
5000
10000
15000
20000
25000
Distance from TSS (bp)
deltaBeta
WT
V600E

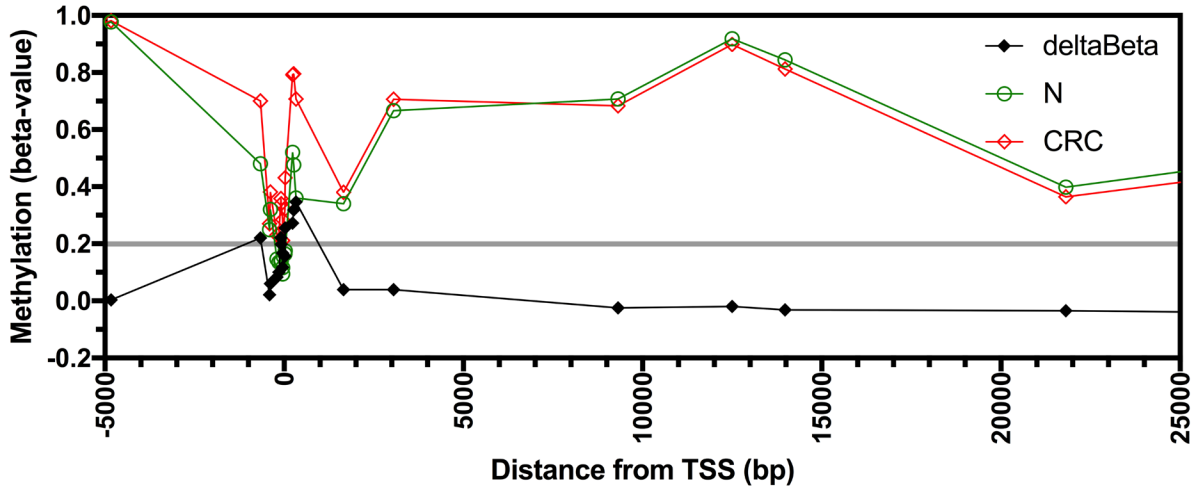


Figure S1: Methylation of AQP1 in normal mucosa and colorectal carcinoma from patients in the combined TCGA-COAD and TCGA-READ datasets. Average beta-values for all available individual probesets, located within the region -5,000 bp upstream and +25,000 bp downstream of the AQP1 transcription start site (TSS), for normal mucosa (N, n = 32) and colorectal carcinoma (CRC, n = 317). Differential methylation (deltaBeta) was calculated by subtracting the average beta-value for N from CRC for each individual probeset.

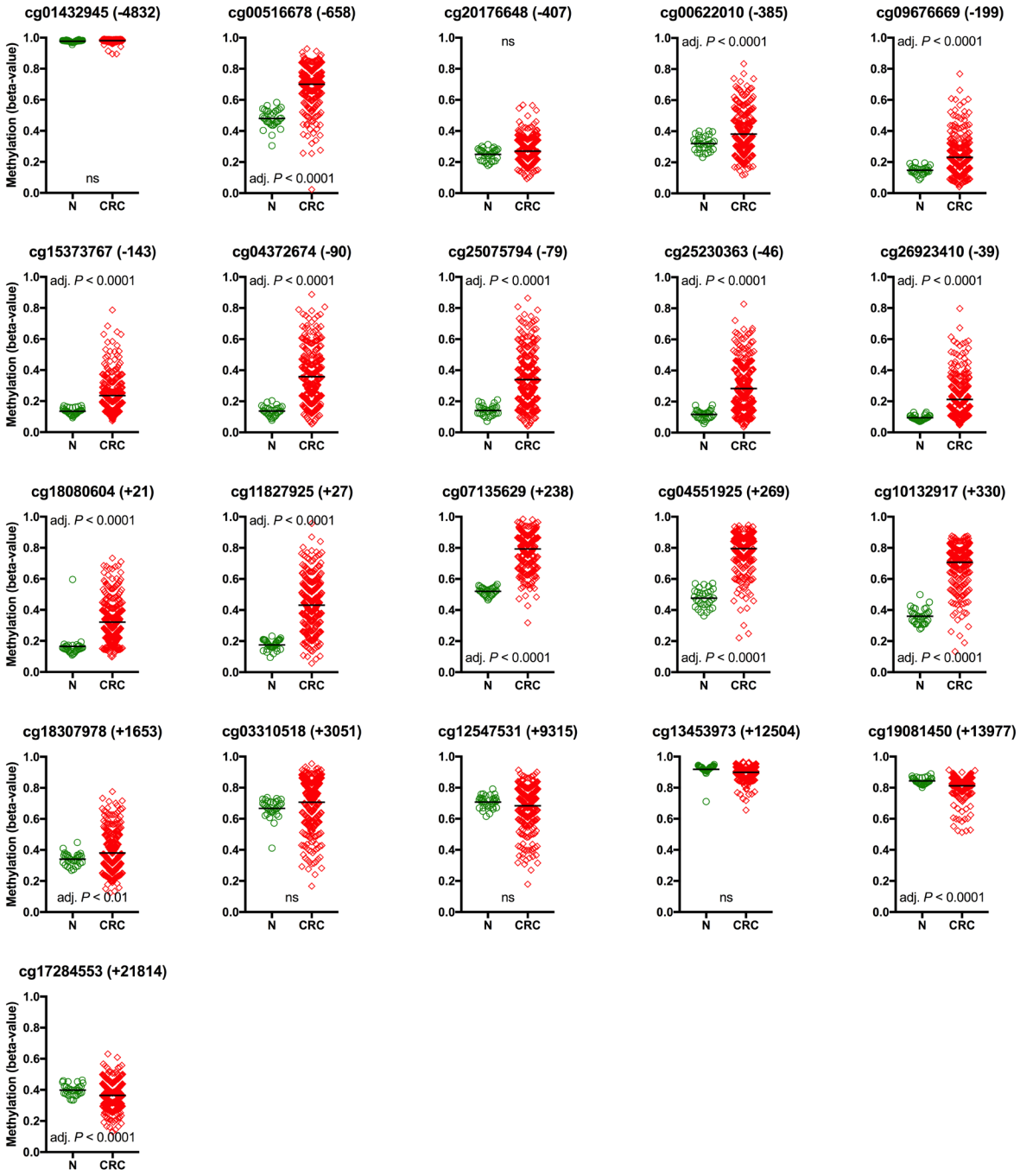


Figure S2: Methylation of individual probesets for normal mucosa and colorectal carcinoma from patients in the combined TCGA-COAD and TCGA-READ datasets. Beta-values for colorectal carcinoma (CRC, n = 317) and normal mucosa (N, n = 32) for all individual probesets located in the region from -5,000 to +25,000 bp of the AQP1 transcription start site. Horizontal bars represent average beta-value for either N or CRC. Comparison between N and CRC were considered statistically significant when the adjusted *P* value (adj. *P*) for the unpaired Welch's t-test with Bonferroni correction for multiple comparisons was < 0.05. ns, not significant.

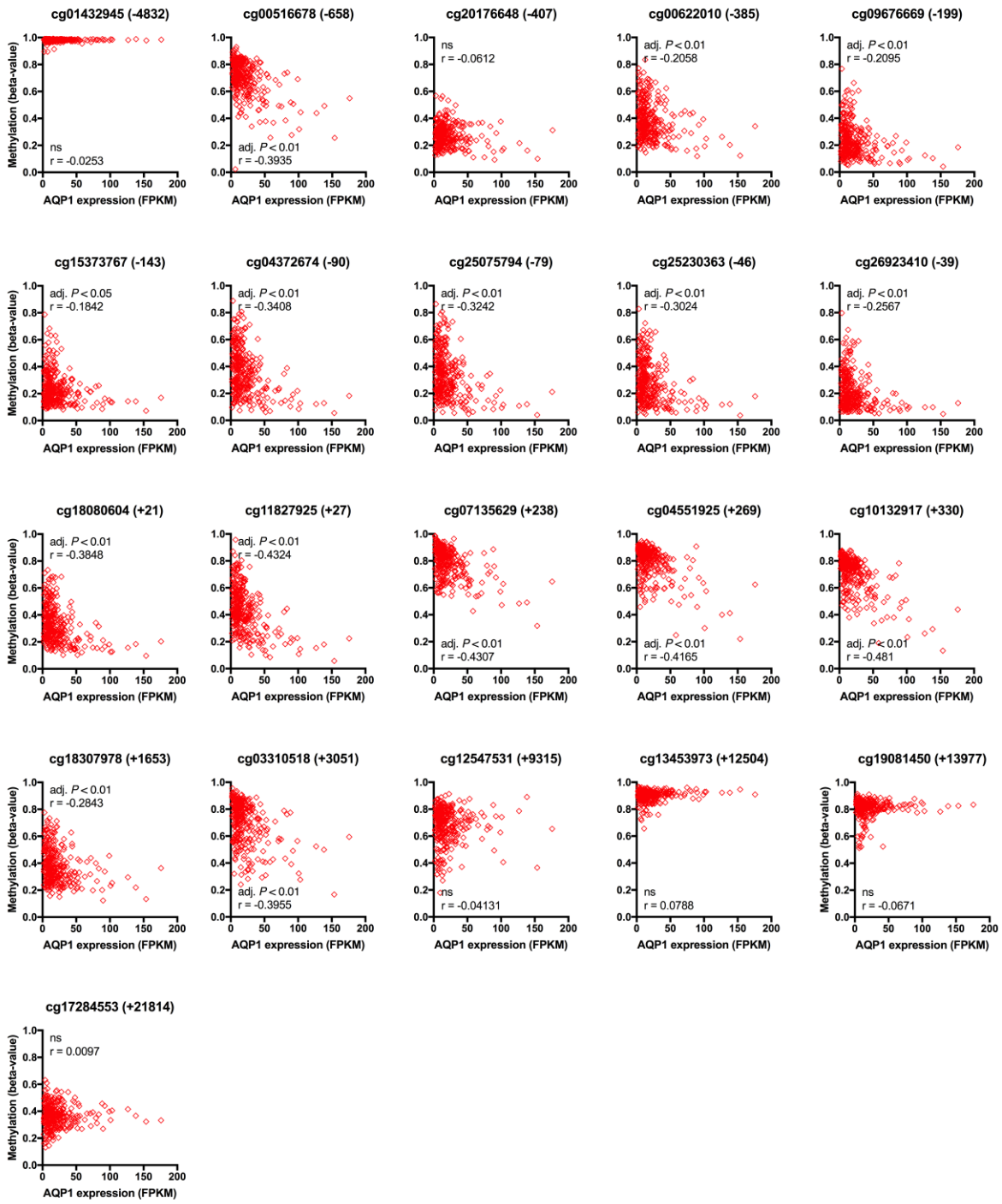


Figure S3: Correlations between AQP1 transcript expression and promoter methylation. RNAseq expression data reported as median number of fragments per kilobase of exon per million reads (FPKM) and HM450 methylation data reported as beta-values for all individual probesets located in the region from -5,000 to +25,000 bp of the AQP1 transcription start site. Expression and methylation data were available for 317 CRC. Correlations between expression and methylation were considered statistically significant when the adjusted *P* value (adj. *P*) for the Spearman correlation coefficient (r) with Bonferroni correction for multiple comparisons was < 0.05. ns, not significant.

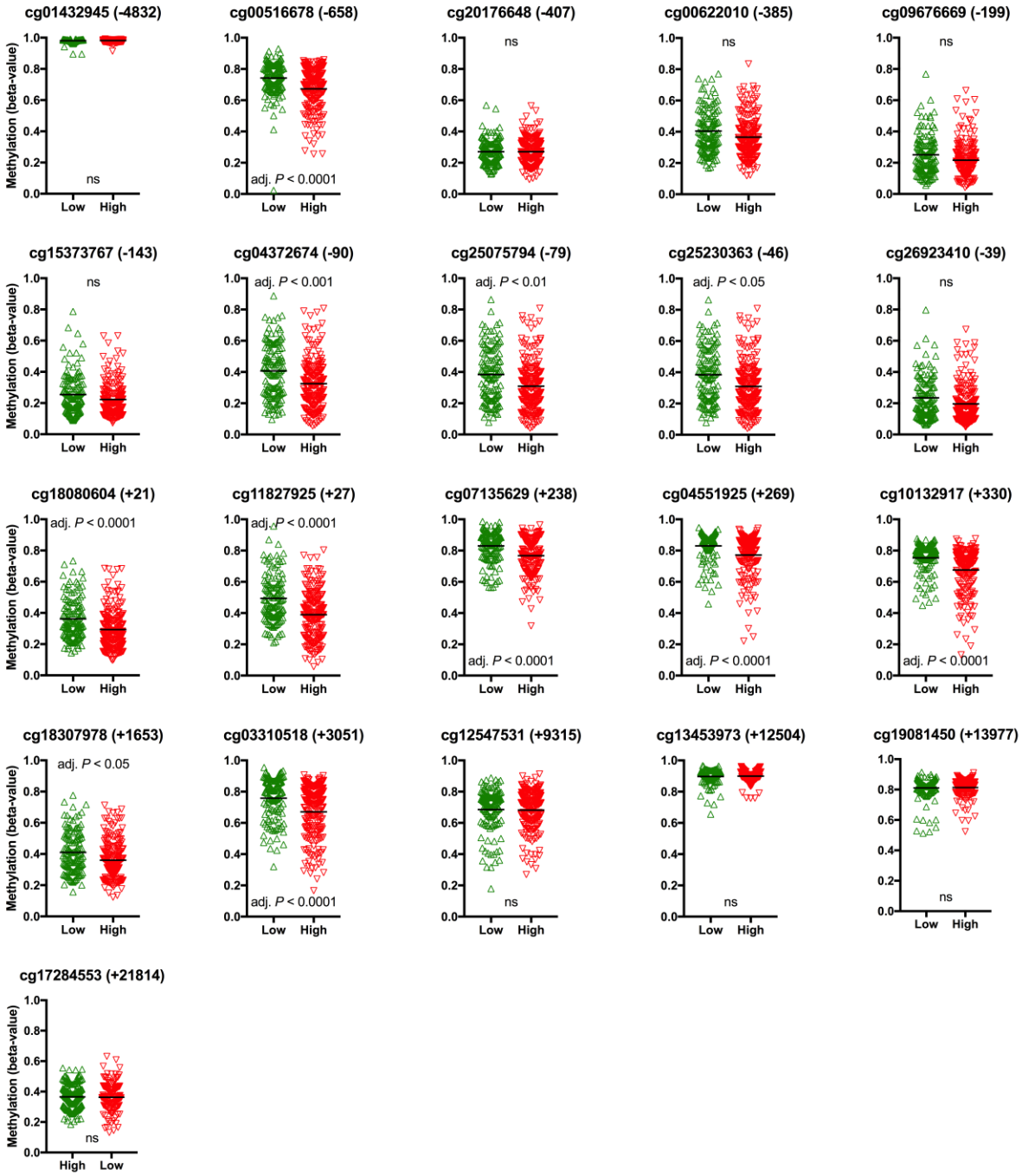


Figure S4: Methylation of individual probesets in low and high AQP1 expressing colorectal carcinoma from patients in the combined TCGA-COAD and TCGA-READ datasets. Beta-values for AQP1 low (n = 127) and high (n = 190) expressing CRC for all individual probesets located in the region from -5,000 to +25,000 bp of the AQP1 transcription start site. Horizontal bars represent average beta-value for either AQP1 low or high expressing CRC. Comparison between AQP1 low and high were considered statistically significant when the adjusted *P* value (adj. *P*) for the unpaired Welch's t-test with Bonferroni correction for multiple comparisons was < 0.05. ns, not significant.

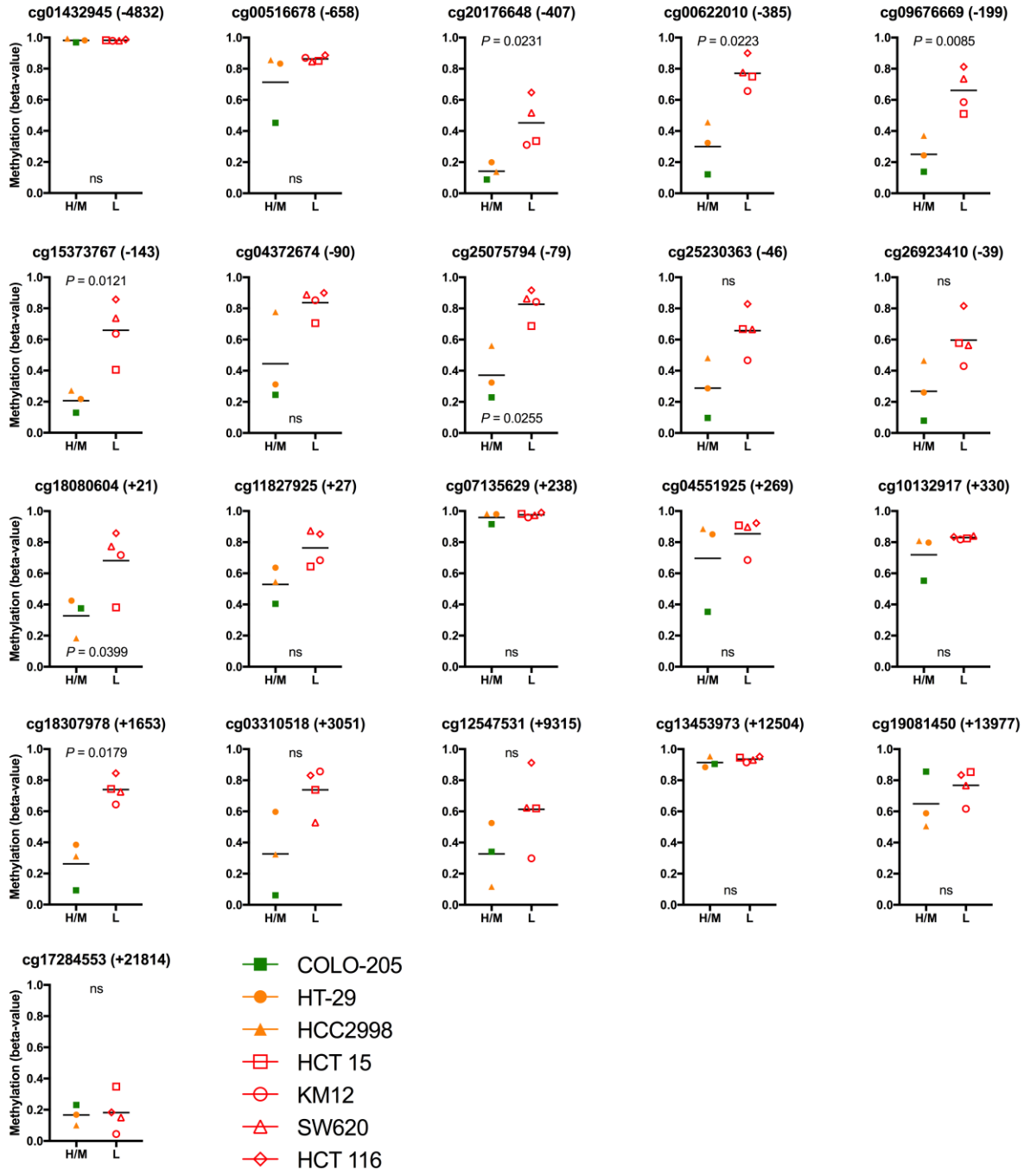


Figure S5: Methylation of individual probesets for colon cancer cell lines in the NCI60 panel. Cell lines were stratified on the basis of AQP1 expression, with COLO-205, HT-29, and HCC2998 defined as high to moderate (H/M), and HCT 15, KM12, SW620 and HCT 116 as low (L). Horizontal bars represent average beta-value for either AQP1 H/M or L expressing CRC. Comparisons between H/M and L AQP1 expressing cell lines were considered statistically significant when the *P* value for the Welch's unpaired t-test was < 0.05. ns, not significant.

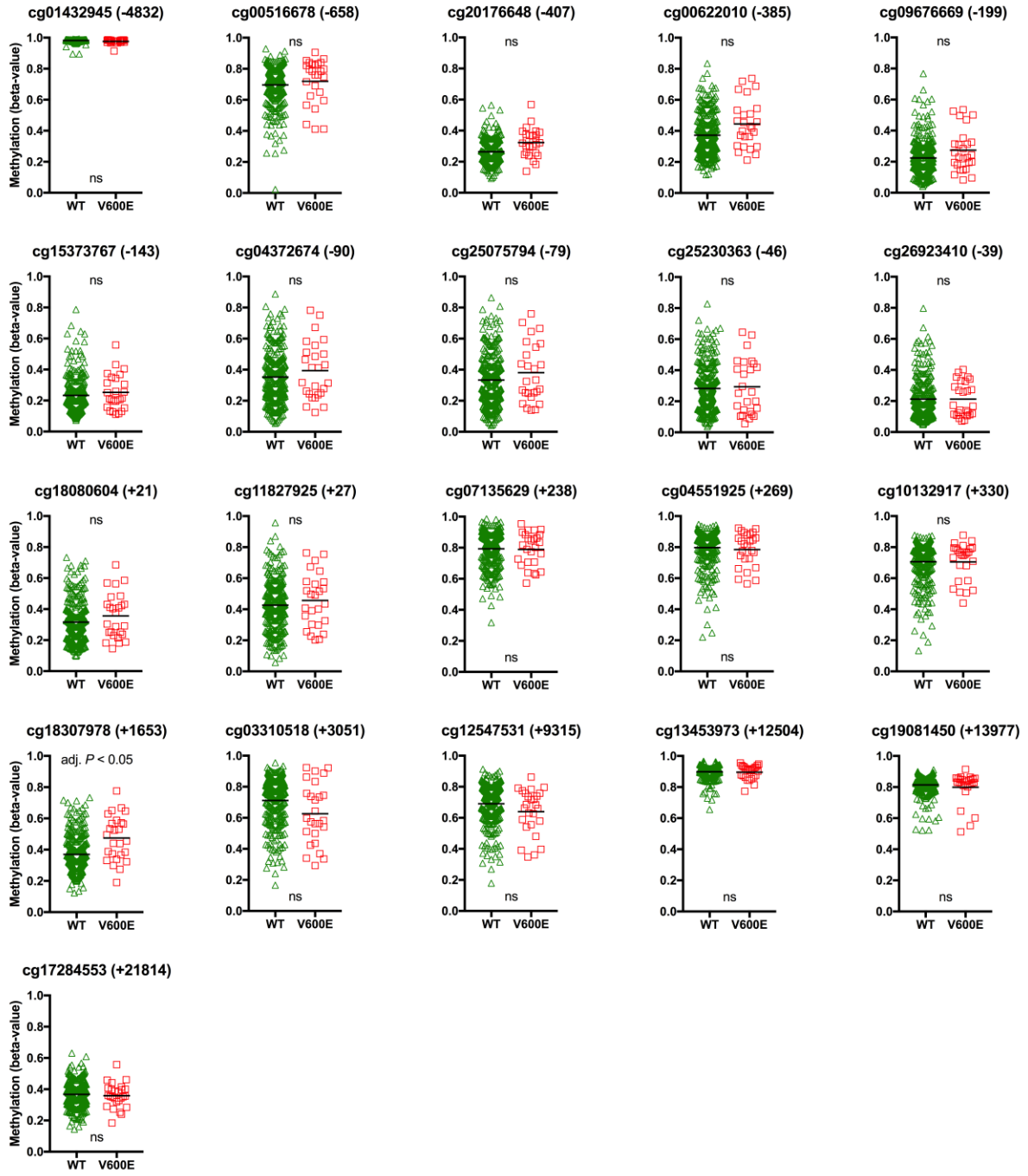


Figure S6: Methylation of individual probesets for colorectal carcinoma from the combined TCGA-COAD and TCGA-READ datasets with either wildtype or *BRAF*^{V600E} mutation. Beta-values for all individual probesets located in the region from -5,000 to +25,000 bp of the AQP1 transcription start site, and *BRAF* mutation status for the corresponding sample were obtained from TCGA Research Network (http://cancergenome.nih.gov/). Horizontal bars represent the average beta-value for either wildtype (WT) or *BRAF*^{V600E} mutation (V600E). Comparisons between WT and V600E were considered statistically significant when the adjusted *P* value (adj. *P*) for the unpaired Welch's t-test with Bonferroni correction for multiple comparisons was < 0.05. ns, not significant.

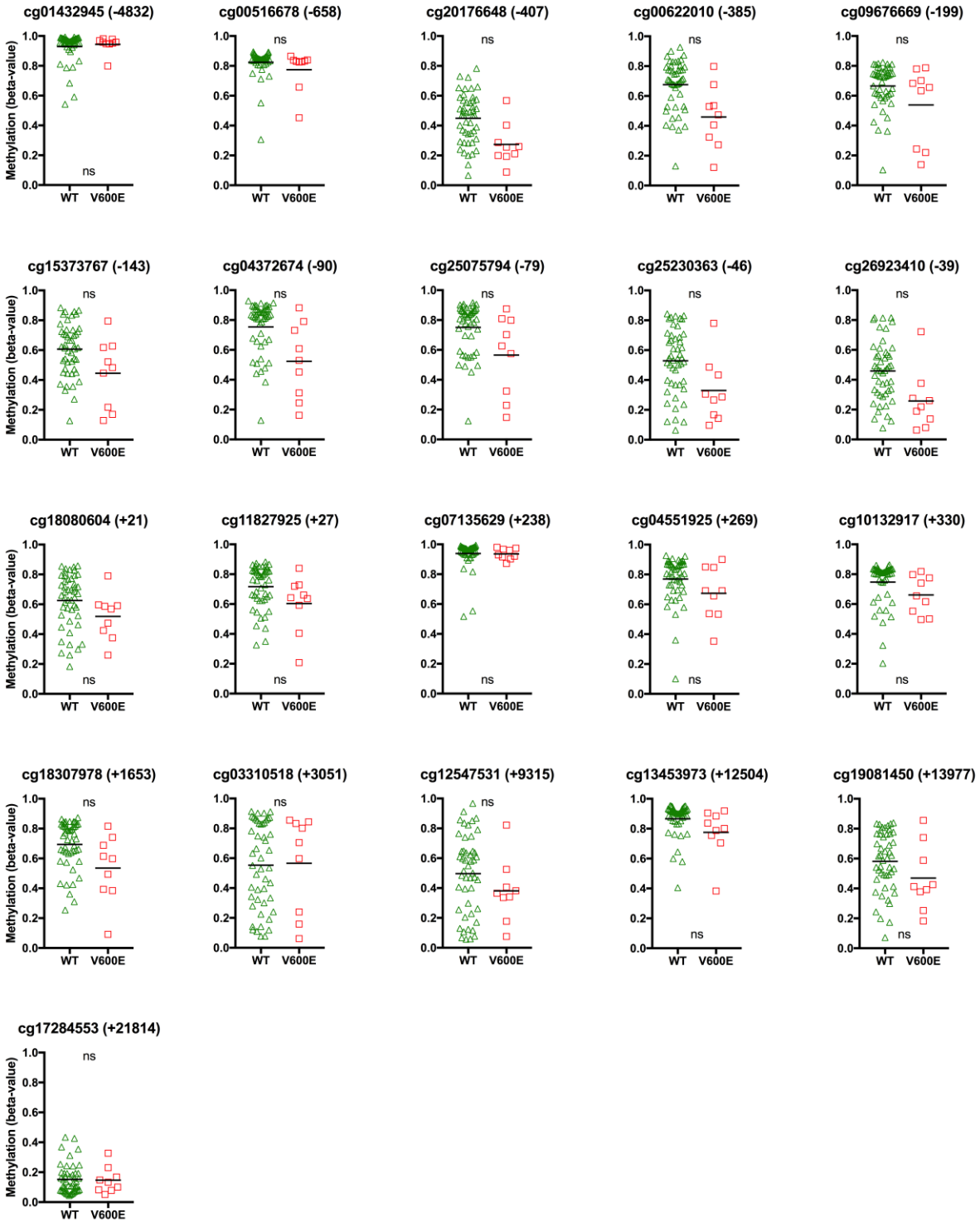


Figure S7: Methylation of individual probesets for cell lines in the NCI60 panel with either wildtype or BRAF^{V600E} mutation. Beta-values for all individual probesets located in the region from -5,000 to +25,000 bp of the AQP1 transcription start site were analysed in the GEO Dataset GSE49143. *BRAF* mutation status for each of the cell lines was obtained from COSMIC (http://cancer.sanger.ac.uk/cosmic). Horizontal bars represent the average beta-value for either wildtype (WT) or *BRAF*^{V600E} mutation (V600E). Comparisons between WT and V600E were considered statistically significant when the adjusted *P* value (adj. *P*) for the

unpaired Welch’s t-test with Bonferroni correction for multiple comparisons was < 0.05. ns, not significant.

Table S1: Clinicopathological features of the 590 colorectal carcinoma patients from the combined TCGA-COAD and TCGA-READ datasets.

Feature	Number	%
All CRC	590	100
Gender		
Female	273	46.3
Male	317	53.7
Age, years		
< 50	71	12.0
≥ 50 ≤ 65	189	32.0
> 65	328	55.6
NR	2	0.3
Stage		
I	102	17.2
II	211	35.8
III	172	29.2
IV	85	14.4
NR	20	3.4
Site of primary		
Right-sided	209	35.1
Left-sided	199	33.4
Rectum	84	14.1
Colon, NOS	98	16.6
Histological subtype		
Mucinous adenocarcinoma (8480/3)	75	12.7
Adenocarcinoma, NOS (8140/3)	495	83.9
Adenocarcinoma in tubulovillous adenoma (8263/3)	8	1.4
Tubular adenocarcinoma (8211/3)	5	0.8
Papillary adenocarcinoma, NOS (8260/3)	2	0.3
Adenocarcinoma with mixed subtypes (8255/3)	2	0.3
Carcinoma, NOS (8010/3)	1	0.2
Adenosquamous carcinoma (8560/3)	1	0.2
Adenocarcinoma with neuroendocrine differentiation (8574/3)	1	0.2

NR, not recorded
NOS, not otherwise specified

Table S2: Analysis of AQP1 transcript expression in normal colonic mucosa, adenoma, and colorectal carcinoma from publicly available datasets submitted to Oncomine.

Author, year (reference)	Normal tissue	Normal tissue median expression (range)[1]	Abnormal tissue	Abnormal tissue median expression (range)[1]	Fold change	*P* value
Gaedcke, et al, 2010 (31)	Normal mucosa (n=65)	3.684 (1.968 to 7.802)	Rectal adenocarcinoma (n=65)	2.606 (-0.126 to 4.831)	-2.341	5.13×10^{-14}
Skrzypczak et al, 2010 (26)	Normal epithelia, microdissected (n=10)	3.806 (2.742 to 4.692)	Adenoma epithelia, microdissected (n=5)	-0.768 (-1.051 to -0.72)	-23.356	4.36×10^{-10}
			Colon carcinoma epithelia, microdissected (n=5)	0.318 (0.056 to 0.383)	-11.129	5.38×10^{-9}
	Normal epithelia and lamina propria, microdissected (n=10)	3.202 (1.118 to 3.6)	Adenoma epithelia and lamina propria, microdissected (n=5)	-0.532 (-0.769 to -0.401)	-10.999	4.11×10^{-8}
			Colon carcinoma epithelia and lamina propria, microdissected (n=5)	1.881 (1.426 to 2.046)	-2.196	7.26×10^{-4}
	Normal mucosa (n=24)	4.045 (2.325 to 6.209)	Colorectal adenocarcinoma (n=45)	2.299 (0.634 to 6.089)	-3.259	2.49×10^{-9}
			Colorectal carcinoma (n=36)	3.236 (1.706 to 5.292)	-1.660	0.002

Hong et al, 2010 (35)	Normal mucosa (n=12)	2.931 (1.906 to 3.585)	Colorectal carcinoma (n=70)	2.77 (0.419 to 5.199)	-1.100	0.241
Gaspar et al, 2008 (29)	Normal mucosa (n=22)	-0.846 (-1.295 to 1.117)	Colorectal adenoma epithelia (n=56)	-1.198 (-2.161 to 0.135)	-1.366	0.002
Sabates-Bellver et al, 2007 (27)	Normal mucosa (n=32)	3.492 (2.192 to 4.222)	Colon adenoma (n=25)	1.715 (-0.892 to 3.342)	-3.565	4.08×10^{-11}
			Rectal adenoma (n=7)	0.708 (-2.897 to 3.966)	-5.687	0.009
Ki et al, 2007 (32)	Normal mucosa (n=41)	1.502 (0.351 to 3.513)	Colon adenocarcinoma (n=77)	1.271 (-0.009 to 5.03)	-1.264	0.012
Kaiser et al, 2007 (34)	Normal mucosa (n=5)	1.95 (1.593 to 2.133)	Caecum adenocarcinoma (n=17)	1.541 (0.637 to 3.698)	-1.276	0.051
Zou et al, 2002 (33)	Normal mucosa (n=8)	2.092 (1.303 to 3.245)	Colon carcinoma (n=9)	3.345 (1.22 to 4.154)	1.877	0.014
			Colon adenocarcinoma (n=41)	1.503 (0.114 to 3.698)	-1.202	0.063
			Colon mucinous adenocarcinoma (n=13)	1.878 (0.303 to 2.765)	-1.116	0.236
			Rectal adenocarcinoma (n=8)	2.151 (0.185 to 2.866)	-1.212	0.246
			Rectosigmoid adenocarcinoma (n=10)	2.078 (1.479 to 3.469)	1.111	0.761
			Rectal mucinous adenocarcinoma (n=4)	3.296 (1.797 to 3.39)	2.035	0.036

Notterman et al, 2001 (28)	Normal mucosa (n=18)	2.338 (1.07 to 2.844)	Colon adenocarcinoma (n=18)	1.892 (0.628 to 2.722)	-1.374	0.006
Alon et al, 1999 (30)	Normal mucosa (n=22)	-0.217 (-1.753 to 0.610)	Colon adenocarcinoma (n=40)	-0.392 (-2.689 to 0.907)	-1.149	0.120

[1]Expression of AQP1 reported as log2 median-centred intensity.