Chapter 4

BRAF mutation-specific promoter methylation of *FOX* genes

manuscript in preparation

Eddy H. J. van Roon[1,2,], Robert E. Ernst, Tom van Wezel[2], Hans Morreau[2], Judith M. Boer[1,4, §]

[1]Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands
[2]Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands
[3]Department of Pediatric Oncology, Erasmus MC, Rotterdam, The Netherlands [4]Netherlands Bioinformatics Center [§]Corresponding author

BRAF MUTATION-SPECIFIC PROMOTER METHYLATION OF *FOX* GENES

Abstract

Cancer-specific hypermethylation of (promoter) CpG islands is common during the tumorigenesis of colon cancer. Although associations between certain genetic aberrations, such as *BRAF* mutation and microsatellite instability, and the CpG island methylator phenotype (CIMP) have been found, the mechanisms by which these associations are established are still unclear.

Using differential methylation hybridization on oligonucleotide microarrays, we generated methylation profiles of paired tumor and normal colon. The majority of CpG regions found differentially methylated between *BRAF* mutant and wildtype tumors showed hypermethylation in the mutant cases. Enrichment of several cancer-related pathways, including the PI3 kinase and Wnt signaling pathways, was found. To focus on genes that are silenced in a tumor-specific rather than a lineage-specific manner, we used information on an epigenetic silencing mark in embryonic stem (ES) cells. Among the genes showing *BRAF* mutation-specific promoter methylation but no H3K27^{me3} mark in ES cells were forkhead box (FOX) transcription factors associated with the PI3-kinase pathway as well as *MLH1*, and *SMO*. Epigenetic down-regulation of these targets may contribute to mutationally active BRAF-driven tumorigenesis, explaining its association with aberrant DNA methylation.

Introduction

The CpG island methylator phenotype (CIMP) was introduced in 1999 by Toyota *et al*. to describe a subset of colorectal tumors with high levels of cancer-specific methylation[1]. Subsequent studies regarding (CIMP) in colon cancer described a strong association between this epigenetic phenotype, *BRAF* mutations and microsatellite instability (MSI)[2-8]. As sporadic MSI colon cancer is caused by promoter methylation of a mismatch repair gene (*MLH1*, *MSH2* or *MSH6*) the association between MSI and the high levels of DNA methylation in CIMP is considered a causative one[9, 10]. However, the association between activating *BRAF* mutations and CIMP remains unclear.

The field of epigenetic research has progressed from a candidate-gene to a genome-wide approach which not only provided a plethora of new candidate targets of cancer-specific DNA methylation but a better understanding of transcription regulation by DNA methylation as well.[11]. Using such genome-wide DNA methylation approaches could help identify new targets of *BRAF* mutation-specific promoter methylation. Hinoue *et al*. examined the CIMP- and *BRAF* mutation-specific methylation status of 1,505 CpG sites, located at 807 genes, in 235 primary colorectal tumors and discovered specific methylation of genes mediating various signaling pathways involved in colon cancer tumorigenesis[2]. In this study, we screened 32,171 CpG sites located at 10,537 genes in 19 colon cancer patients to obtain additional insight into the association between *BRAF* mutations and DNA methylation in colon cancer tumorigenesis.

Recent publications have reported a possible pre-marking of cancer-specific hypermethylated genes by the inactivation mark Histone H3 lysine 27 trimethylation (H3K27^{me3}) and binding

of polycomb group member SUZ12 in both ES cells and differentiated normal colon mucosal tissue[12-14]. These studies led to the suggestion that colon cancer cells utilize a pre-existing repression program to target loci for cancer specific promoter methylation[12, 14-16]. However, the presence of such repressive histone modifications at promoters during differentiation from ES to normal colon epithelium suggests that the associated genes are at a transcriptional silent state prior to tumor formation, reducing the relevance of the DNA methylation of pre-marked genes on tumorigenesis. In an attempt to identify biologically relevant *BRAF* mutation-specific promoter methylation, we excluded loci with H3K27^{me3} pre-marking in ES cells from the functional pathway analyses. By both extending the number of screened loci and filtering out pre-marked genes we identified new targets of *BRAF* mutation-specific methylation that could either create a favorable setting for the acquisition of *BRAF* mutations or function as an addition to up-regulation of the RAS-RAF-MEK pathway.

Materials and methods

Patient material

Anonymized tumor and normal fresh-frozen colon mucosa samples were obtained from patients who underwent surgery between 1988 and 2006 at the Leiden University Medical Center (Leiden, The Netherlands) or at the Rijnland Hospital (Leiderdorp, The Netherlands). Age, gender, histology, microsatellite instability (MSI), and *BRAF* V600E status for the 19 patients used for the array profiling are listed in Supplementary Table S1. Prior to DNA isolation, frozen sections were micro-dissected to minimize the presence of normal epithelium and stromal cells. To correct for age-dependent methylation, we used normal mucosa, distant from the tumor, from the same individuals. DNA was isolated by phenol/chloroform extraction and ethanol precipitation from 10-20 sections of 30 μm and yielded 10-50 μg. The present study was approved by the Medical Ethics committee of the LUMC (protocol P01-019). Cases were analyzed following the medical ethical guidelines described in the Code Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences.

BRAF mutation analysis

BRAF V600E mutations were detected using flanking primers that have been previously described[17]. PCR products were purified with the QIAquick PCR Purification kit (Qiagen, Hilden, Germany). Sequencing was performed at the Leiden Genome Technology Center (LGTC, Leiden, The Netherlands) using an ABI 3730 XL (Applied Biosystems, Foster City, CA). Mutational analysis was performed using mutational surveyor (SoftGenetics LLC., State College, PA). Results are summarized in Supplementary Table S1.

Array hybridization

Differential methylation hybridization (DMH) was performed according to Yan *et al.* [18]. DNA (500 ng) was digested with *Mse*I, ligated to linkers, and sequentially digested with two methylation-sensitive restriction enzymes (HpaII and BstUI, New England Biolabs, Ipswich, MA, USA). Digested linker-ligated DNA was used as template for polymerase chain reaction (PCR) amplification (20 cycles) and coupled to fluorescent dyes. Cy5- or Cy3-labeled amplicons, representing methylated DNA fragments derived from tumor and

normal samples, were co-hybridized to the Agilent 244k human CpG island microarrays (Agilent Technologies, Santa Clara, CA, USA) in a dye-swap setup. Detection was done on a G2565BA scanner (Agilent Technologies) and feature extraction using Feature Extraction Software version 9.5.3.1 (Agilent Technologies).

Array data analysis

Non-background corrected data was preprocessed by within-array loess normalization followed by between-array aquantile normalization using limma v3.2.1[19] in R2.10.0[20]. Data was corrected for gene-specific dye bias using R package dyebias v1.4.0[21]. Raw data and preprocessed log2 ratios (tumor versus normal) per probe are available via GEO under accession number GSExxxxx. Probes mapping to the same *Mse*I fragment were expected to show similar hybridization patterns and not be independent. Therefore, we mapped probes to the human genome (UCSC assembly March 2006) cut *in silico* with *Mse*I. Fragments of 150-3000 bp mapping at least one complete probe and containing at least one BstUI or HpaII restriction site (n=32,171) were selected. In total, 195,625 of the 244,000 array probes (80.2%) mapped to such informative fragments, mostly with 1-2 probes per fragment, up to 33. For statistical analysis and visualization, the median log ratio per fragment was used to represent the fragment. Methylation differences between tumor and normal samples and tumor subgroups were analyzed using a linear model in limma v3.2.1[19]. The obtained P-values were corrected for multiple-testing[22] and fragments with a false discovery rate (FDR) <= 0.01 were selected as significant.

MLH1 and CIMP marker methylation

DNA samples (500 ng) were converted using the EZ DNA methylation Gold bisulphite kit (Zymo Research). For validation of methylation changes we performed a methylation-specific PCR (MSP) on the *MLH1* promoter using primers previously described[23] (Supplementary Table S1). Methylation of previously described CIMP markers:MINT1, MINT2, MINT12, MINT31, *RIZ1* and *TIMP3* was determined by MSP, while MINT27 and Megalin methylation was determined by Combined Bisulfite Restriction Analysis (COBRA)[6, 24]. Amplifications were carried out in a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA) using AmpliTaq Gold PCR buffer and enzyme (Applied Biosystems, Foster City, USA). Amplified bands were visualized on a 2% agarose gel.

Exploratory data analysis

Differentially methylated fragments were compared to publicly available data containing chromosomal regions identified in chromatin immunoprecipitation using antibodies against $H3K27^{me3}$, $H3K4^{me3}$, CTCF and SUZ12 in ES cells followed by high-throughput sequencing[25-27]. By using the sqldf R package (version 0.3-5), we determined overlap of at least 20 bp between CpG fragments represented on the array and these regions. Enrichment of chromatin domains among the differentially methylated fragments was calculated by χ-squared test. Functional annotation clustering was performed in Panther 6.0[28]. Filtering of the differential methylation datasets by $H3K27^{me3}$ in ES cells using the dataset from Zhao *et al*.[27] was performed in R using the sqldf package.

Results

Colon cancer-specific CpG island methylation

We identified 1770 CpG-rich fragments with significant methylation differences between tumor and paired normal colon. Of these, 1234 fragments were associated with 816 genes, of which 531 were localized to gene promoters (Supplementary Table S2). As expected, CpG islands were mostly found hypermethylated in tumors (78.8%)[11].

We compared our results with those of Irizarry et al[11] who described 2707 cancer-specific differentially methylated regions (cDMRs) based on the comparison of 13 colorectal cancer tumor-normal pairs. Of the described cDMRs, 1203 overlapped with our CpG island array fragments, of which 282 (23%) were also differentially methylated between tumor and normal in our analysis. This overlap is reasonable considering the different, modest sized patient groups, and different experimental approaches.

CIMP-specific methylation

Next, we compared the tumor-normal methylation ratios between different groups of patients. Between CIMP-positive (n=11) and CIMP-negative (n=8) patients 749 CpG-rich fragments showed methylation changes, of which 85.6% had a higher tumor-normal methylation ratio in the CIMP-positive group. Of these fragments, 589 were associated with 508 genes, of which 244 were localized to gene promoters (Supplementary Table S3). In 8 out of 11 CIMP-positive tumors, promoter methylation of *MLH1*, the cause of microsatellite instability (MSI) in sporadic colon cancer, was observed which was consistent with methylation-specific PCR (Supplementary Table S1). We conclude that the hypermethylation in specific genomic regions used to define CIMP[6] is associated with methylation changes throughout the genome.

BRAF mutation-specific methylation

Activating *BRAF* mutations have been associated with high levels of CpG island methylation and MSI in colon cancer[2-8]. To investigate this association we compared the tumor-normal methylation ratio profiles of *BRAF* wildtypes (n=11) with those containing the *BRAF* V600E mutation (n=8). We identified 758 fragments with a *BRAF* mutation-specific methylation change, of which 96.3% had a higher tumor-normal methylation ratio in the *BRAF* mutant group. Out of these 758 fragments 579 were associated with 479 genes, of which 229 were localized to gene promoters (Supplementary Table S4).

Since *BRAF* mutations and CIMP co-occurred in eight samples, there was a high level of overlap between CIMP- and *BRAF* mutation-specific methylation changes (Figure 1A). Comparable levels of overlap were found focusing on promoter regions only (data not shown).

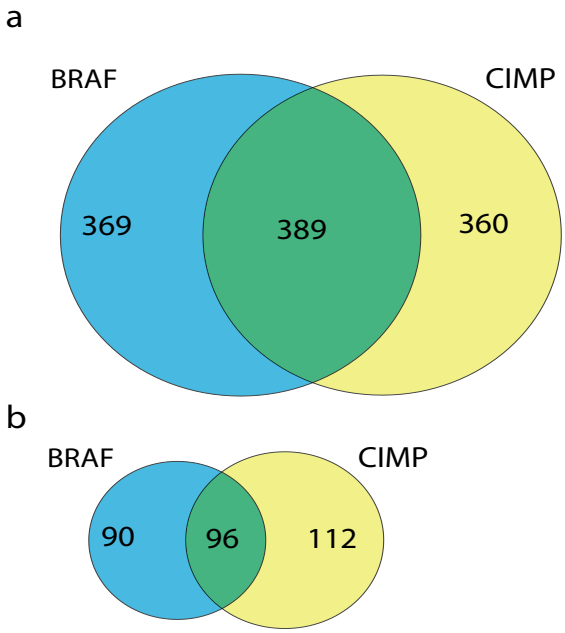

Figure 1- Proportional Venn diagrams showing the overlap between *BRAF* mutation- (blue) and CIMP-specific (yellow) methylation changes for all CpG-rich fragments (**A)** and promoter fragments filtered for H3K27^{me3} binding in ES cells (**B**).

Regions with tumor, CIMP and BRAF mutation-associated methylation changes are enriched for SUZ12 and H3K27^{me3} while depleted for CTCF and H3K4^{me3}

Regions binding the polycomb repressor complex 2 (PRC2) component SUZ12 in ES cells[29] were found to be enriched among the loci differentially methylated between colon cancer and normal colon (Table 1). The histone mark H3K27^{me3} is mediated by the PRC2 complex[29], and the two marks have been reported to be highly correlated[13]. Enrichment of ES cell H3K27^{me3} binding regions among the fragments with colon cancer-associated methylation changes was therefore expected and indeed observed. Similarly, fragments with CIMP- and *BRAF* mutation-associated differential methylation changes were also highly enriched for regions binding SUZ12 and H3K27^{me3} in ES cells (Table 1). Additionally, sites binding CTCF and the active chromatin mark H3K4^{me3} were underrepresented among the differentially methylated fragments. Interestingly, although all colon cancer-, CIMP- and *BRAF* mutation-specific fragments are underrepresented for H3K4^{me3}, this depletion is most evident for *BRAF* mutation-specific fragments.

After exclusion of fragments with H3K27^{me3} pre-marking in ES cells the overlap between CIMP- and *BRAF* mutation-specific methylation changes for all loci (Figure 1A) and promoters (Figure 1B) remained highly significant. Despite this high level of overlap approximately 50% of *BRAF* mutation-specific methylation changes showed no overlap with CIMP. In our functional analysis we focused on all promoter fragments with *BRAF* mutation-specific methylation changes regardless of overlap with CIMP.

	Total	SUZ12	p-val*	H3K27me3	p-val	CTCF	p-val	H3K4me3	p-val
Colon tumor fragments	1770	540(30.5%)	0	561(31.7%)	0	379(21.4%)	0	711(40.2%)	0.00001285
Remaining fragments	30401	3447(11.3%)		3336(11%)		10137(33.3%)		13835(45.5%)	
CIMP-specific fragments	749	160(21.4%)	0	169(22.6%)	0	183(24.4%)	0.0000011	300(40.1%)	0.0045882
Remaining fragments	31422	3827(12.2%)		3728(11.9%)		10333(32.9%)		14246(45.3%)	
BRAF-specific fragments	758	171(22.6%)	0	180(23.7%)	0	195(25.7%)	0.00003543	169(22.3%)	0
Remaining fragments	31413	3816(12.1%)		3717(11.8%)		10321(32.8%)		14377(45.8%)	

* p-val: P-values of the χ-square test

Table 1- Number of fragments with colon tumor-, CIMP- and *BRAF* mutation-specific methylation changes containing overlap with indicated chromatin marks in ES cells.

BRAF mutation-specific promoters (125)	Associated genes	Exp hits‡	Hits	p-val†
Hedgehog signaling pathway	*SMO ; GSK3A* ; CREBBP**	0.16	3	5.61E-04
PI3-kinase pathway	*FOXB1 ; FOXB2 ; FOXD3 ; CCND1 ; GSK3A**	0.72	5	8.49E-04
Insulin/IGF pathway-protein kinase B signaling Cascade	*FOXB1 ; FOXB2 ; FOXD3 ; GSK3A**	0.56	4	2.52E-03
Wnt signaling pathway	*NKD2* ; GNG4 ; CCND1 ; GSK3A* ; CREBBP* ; AXIN1 ; LEF1*	1.99	7	4.05E-03
Transcription regulation by bZIP transcription factor	*MTERF ; CREBBP* ; TAF7*	0.33	3	4.71E-03

*Asterisks mark the targets with low log2 in the *BRAF* wildtype group, suggesting that additional mechanisms such as genomic loss may play a role

† p-val: P-values of the binomial test between the *BRAF* mutation-specific gene list and the reference gene list

‡Exp hits: Expected number of hits by chance in the reference gene list

Table 2 - Pathways enriched with genes showing *BRAF* mutation-associated promoter methylation after exclusion of ES-cell H3K27^{me3} binding promoter fragments.

BRAF mutation-associated methylation pathway analysis

To identify biological pathways affected by *BRAF* mutation-associated gene methylation, we used 186 promoter regions that did not bind H3K27^{me3} in ES cells representing 125 genes after exclusion of duplicates and annotation by Panther 6.0. We found five significantly enriched pathways (P-val < 0.01, Table 2) containing 13 unique genes (Table 2).

With seven genes, the WNT pathway contained the most *BRAF* mutation-specific methylation changes (Table 2). However, the tumor-normal log2 ratios (Figure 2) of *AXIN1*, *CREBBP*, *GSK3A* and *NKD2* in the *BRAF* wildtype samples were low (-0.26 median, 0.12 standard deviation) compared to those in the *BRAF* mutated samples (-0.02 median, 0.12 standard deviation). While this could indicate tumor hypomethylation in *BRAF* wildtype samples compared to normal and *BRAF* mutated samples, the high level of chromosomal instability among *BRAF* wildtype samples suggests that copy-number loss is the most plausible explanation for this. To filter for this phenomenon we excluded fragments with a log2 ratio below one standard deviation of the median log2 ratio of all *BRAF* mutation-specific fragments in the *BRAF* wildtype group. A significant increase in the *BRAF* mutant log2 ratios compared to those of the *BRAF* wildtypes (approximately 0), indicate *BRAF* mutation-specific hypermethylation in these colon cancer samples (Figure 2). Upon filtering nine of the pathway associated genes remained (*SMO, FOXB1, FOXB2, FOXD3, CCND1, GNG4, LEF1, MTERF, TAF7*) and the PI3 kinase pathway was the only statistically significant enriched (P-val: 5.5E-03) pathway. Interestingly, besides promoter methylation of PI3 kinase pathway-associated forkhead box (FOX) genes we identified promoter methylation of three other FOX transcription factors: *FOXA1*, *FOXC1* and *FOXF1*. However, these promoters were bound by H3K27^{me3} and were excluded from our pathway analysis.

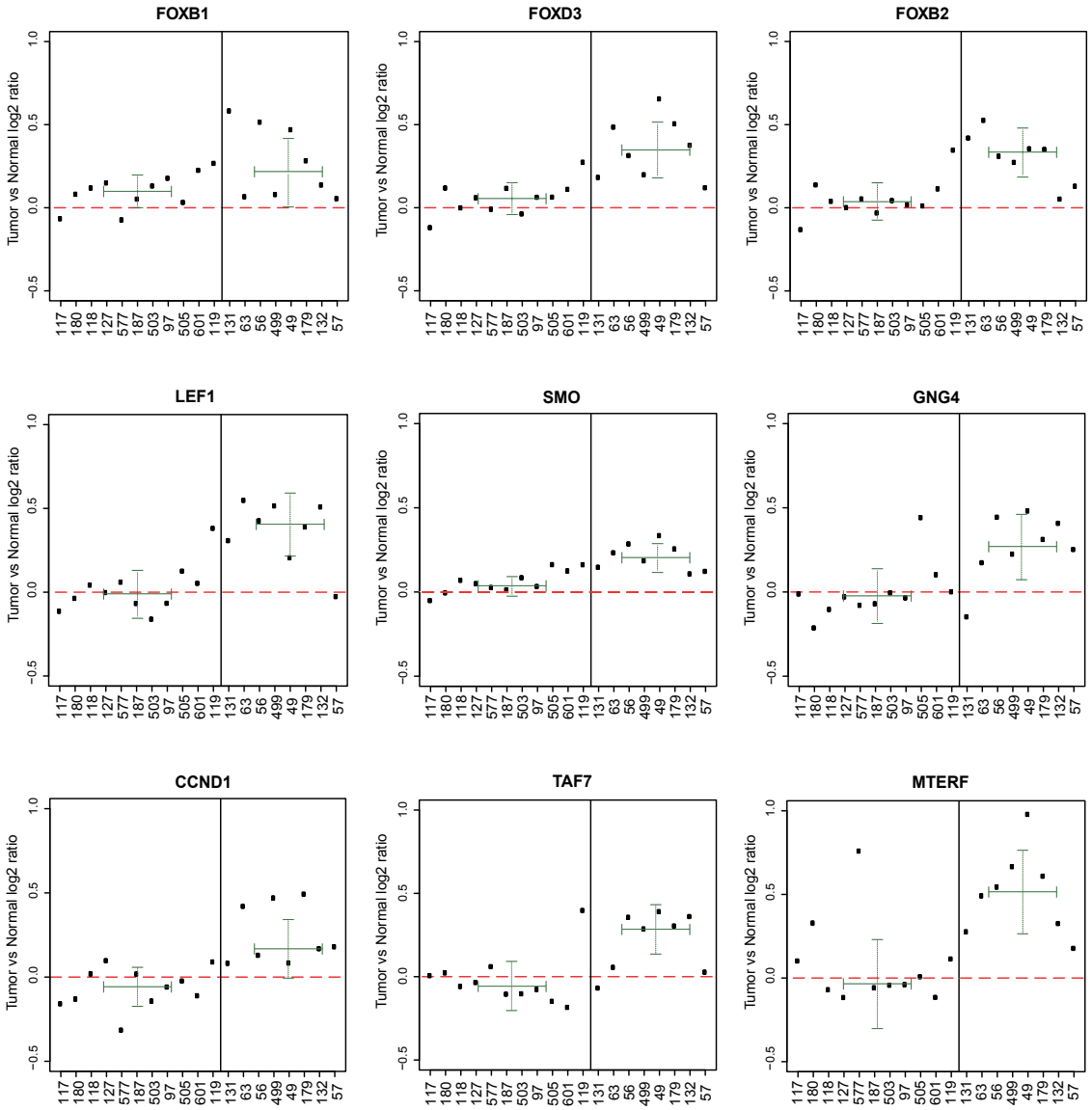

Figure 2 - Scatter plots for 9 unique genes with *BRAF* mutation-specific promoter methylation causing pathway enrichment. The Y-axis represents the tumor-normal log2 ratio for the median probe per fragment. Sample IDs are given below the X-axis with *BRAF* wildtypes left of the black line and *BRAF* mutants on the right. Median log2 ratios and standard deviations (dotted lines) for the *BRAF* wt and *BRAF* mutant groups are given in dark green.

Discussion

In this study we extended the number of screened CpG loci compared with previous studies performed in context of *BRAF* mutations to identify new *BRAF* mutation-specific methylation changes. This association between DNA methylation and activating *BRAF* mutations in colon cancer has been identified in multiple studies[2-8]. Here, we attempted to identify additional targets of *BRAF* mutation-specific DNA methylation that could provide a favorable context to either obtain a *BRAF* mutation or to attain the full potential of RAS-RAF-MEK induced proliferation provided by this activating mutation. Identified targets of promoter methylation showing pre-marking by H3K27^{me3} in ES cells were excluded to filter out methylation changes with minimal expected effects on transcription and thereby tumorigenesis[13]. We showed high levels of overlap between CIMP- and *BRAF* mutation-specific methylation changes which remained after filtering out pre-marked loci. Although Rada-Iglesias *et al.* showed a higher pre-marking of colon cancer-specific DNA methylation by H3K27^{me3} binding in normal colon epithelium compared to ES cells, we were restricted to using ES cell data due to incompatibility between data formats in our analyses[13]. Interestingly the promoter region of *MLH1*, found methylated in both a CIMP- and *BRAF* mutation-specific manner, was not filtered out. Therefore, *MLH1* promoter methylation, the cause of sporadic MSI colon cancer, is not established through utilization of a pre-existing repressive program in ES cells.

The study by Hinoue *et al.*[2] described *BRAF* mutation-specific DNA methylation of 60 genes in a comparison of 1505 CpG sites between 33 *BRAF* mutated tumors and 202 *BRAF* wildtype tumors. The identification of promoter methylation of the mediator of BRAFV600E-induced senescence, *IGFBP7*, led them to suggest that this epigenetic silencing provides a favorable context for the acquisition of *BRAF* mutations[2, 30]. Despite differences in experimental techniques and coverage, ten genes overlapped with our set of *BRAF* mutation-specific methylated fragments, including the RAS-RAF hyperactivation-associated *BMP3*, receptor kinases *EPHA3* and *FLT3* as well as the Hedgehog (Hh) signaling protein *SMO*. However, no overlap was found for the mediator of RAS-RAF oncogene-induced senescence, *IGFBP7*, possibly due to lack of *IGFBP7* promoter area coverage in our assay. Additionally, *BMP3* and *EPHA3* were pre-marked by H3K27^{me3} in our analysis suggesting minimal impact on gene expression and tumorigenesis.

We initially identified enrichment of five cancer-associated pathways by *BRAF* mutation-specific promoter methylation of 9 unique genes. Our analysis took into account copy number changes and filtered for this which could improve the reproducibility of DMH based assays[18, 31]. Exclusion of these loci resulted in the PI3-kinase pathway as the only pathway enriched in our analysis.

Among the four genes enriched in this pathway are the FOX transcription factors *FOXD3*, *FOXB1* and *FOXB2*. A recent study described FOXD3 as a p53 and p21^{cip1}-dependent negative cell cycle regulator which is suppressed by activated BRAF in melanoma cells[32]. Down-regulation of FOXD3 levels by promoter methylation could provide a favorable setting for either acquisition of a *BRAF* mutation or proliferation by RAS-RAF-MEK over-activation in colon cancer, similar to *IGFBP7*[2]. Interestingly, the FOXO transcription factors have been described as mediators of p21^{cip}-dependent BRAF induced senescence as well, indicating that multiple FOX genes are involved in this process[33]. We identified additional FOX genes with *BRAF* mutation specific promoter methylation that were not annotated as part of

the PI3-kinase pathway in our analysis as they were pre-marked by H3K27^{me3} in ES cells: *FOXA1*, *FOXC1* and *FOXF1*. However, the promoters of these genes were also pre-marked with H3K4me^{3} indicating possible tissue-specific expression of these genes. All three are targets of inactivation in breast cancer and both *FOXC1* and *FOXF1* subjected to promoter methylation[34-36]. Most intriguing is the description of FOXF1 as an inducer of G1-S and S-G2 cell cycle arrest, indicating a possible role in oncogene induced senescence[35]. Additional research is required to determine the role of these FOX genes in colon cancer-associated oncogene induced senescence and what the impact of their promoter methylation is on this mechanism. Finally, research into the sequence of such events is required to provide a better insight in the association between activating *BRAF* mutations and DNA methylation in colon cancer.

Acknowledgements

The authors would like to thank Michiel van Galen (Leiden Genome Technology Center Leiden, The Netherlands) for support with mapping fragments, and Philip Lijnzaad (Department of Physiological Chemistry, University Medical Center Utrecht, Utrecht, The Netherlands) for support with the dyebias R package.

Supplementary data

Supplementary Table S1 - Sample information. Gender (indicated by M for male and F for female), Age, Histology (indicated by Ad for adenoma and Ca for carcinoma), *MLH1* promoter methylation status and *BRAF* mutation status is given.

Patient ID	Gender	Age	Histology	*MLH1* methylation	*CIMP* status	*BRAF* V600E
117	M	69	Ad	U	-	wt
180	F	86	Ad	U	-	wt
118	F	69	Ad	U	-	wt
127	F	87	Ad	U	-	wt
577	M	32	Ad	U	-	wt
187	F	61	Ca	U	-	wt
503	F	63	Ca	U	-	wt
97	F	68	Ca	U	-	wt
131	F	71	Ca	U	+	mut
505	F	83	Ca	U	+	wt
63	F	57	Ca	U	+	mut
57	F	75	Ca	pM	+	mut
601	F	83	Ad	M	+	wt
56	M	75	Ca	M	+	mut
499	F	69	Ca	M	+	mut
119	F	76	Ca	M	+	wt
49	F	80	Ca	M	+	mut
179	M	90	Ca	M	+	mut
132	F	64	Ca	M	+	mut

Supplementary Table S2 - **Fragments with tumor-specific methylation changes**. Genomic location is provided by chromosome number (Chr) and the start (start) and end (end) position in the human genome build 18 (UCSC assembly: March 2006) basepair number. Annotation as promoter, divergent promoter or otherwise and the associated gene (UniGene and EntrezGene numbers) in case of promoter or intergenic (annotated as inside) is given as well as fragments length (length), number of DNA methylation-specific restriction sites (BstUI and HpaII), number of probes located in the specific fragment (Probes) and pre-marking by H3K27me^{3} in ES cells. Available upon request

Supplementary Table S3 - Fragments with CIMP-specific methylation changes. Genomic location is provided by chromosome number (Chr) and the start (start) and end (end) position in the human genome build 18 (UCSC assembly: March 2006) base pair number. Annotation as promoter, divergent promoter or otherwise and the associated gene (UniGene and EntrezGene numbers) in case of promoter or intergenic (annotated as inside) is given as well as fragments length (length), number of DNA methylation-specific restriction sites (BstUI and HpaII), number of probes located in the specific fragment (Probes) and pre-marking by H3K27me^3 in ES cells. Available upon request

Supplementary Table S4 - Fragments with BRAF mutation-specific methylation changes. Genomic location is provided by chromosome number (Chr) and the start (start) and end (end) position in the human genome build 18 (UCSC assembly: March 2006) base pair number. Annotation as promoter, divergent promoter or otherwise and the associated gene (UniGene and EntrezGene numbers) is given as well as fragments lengths, number of DNA methylation-specific restriction sites (BstUI and HpaII), number of probes located in the specific fragment (Probes) and pre-marking by H3K27me^3 in ES cells. Available upon request

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