Methylation and Gene Expression of *BCAT1* and *IKZF1* in Colorectal Cancer Tissues

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ABSTRACT: The genes *BCAT1* and *IKZF1* are hypermethylated in colorectal cancer (CRC), but little is known about how this relates to gene expression. This study assessed the relationship between methylation and gene expression of *BCAT1* and *IKZF1* in CRC and adjacent non-neoplastic tissues. The tissues were obtained at surgery from 36 patients diagnosed with different stages of CRC (stage I n = 8, stage II n = 13, stage III n = 10, stage IV n = 5). Methylated *BCAT1* and *IKZF1* were detected in 92% and 72% CRC tissues, respectively, with levels independent of stage (*P* > .05). In contrast, only 31% and 3% of non-neoplastic tissues were methylated for *BCAT1* and *IKZF1*, respectively (*P* < .001). The *IKZF1* messenger RNA (mRNA) expression was significantly lower in the cancer tissues compared with that of non-neoplastic tissues, whereas the *BCAT1* mRNA levels were similar. The latter may be due to the *BCAT1* polymerase chain reaction assay detecting more than 1 mRNA transcript. Further studies are warranted to establish the role of the epigenetic silencing of *IKZF1* in colorectal oncogenesis.

KEYWORDS: Methylation, *BCAT1*, *IKZF1*, expression, colorectal cancer

Introduction

Cancer is a result of genetic and epigenetic modifications.[1] Accumulation of these abnormalities leads either to gain of function through the activation of oncogenes or loss of function through the deactivation of tumour suppressor genes.[2] A growing body of evidence implies that epigenetic changes, such as hypermethylation or hypomethylation, are much more frequent than genetic alterations in colorectal cancer (CRC).[3,4] Although hypomethylation can be observed throughout the genome, hypermethylation is commonly observed in CpG islands embedding gene promoter regions.[5]

An increasing number of genes with aberrant methylation have been found to be involved in CRC development.[6,7] Branched chain aminotransaminase 1, *BCAT1*, and IKAROS family zinc finger 1, *IKZF1*, are 2 such genes found to be hypermethylated with high frequency in both adenoma and CRC tissue.[6] *IKZF1* plays critical roles in regulating numerous biological events including immune system function and hematopoietic proliferation and differentiation.[8–10] More importantly, *IKZF1* is involved in regulating cell-to-cell interactions through mediating the activities of Notch signaling pathway.[11] *BCAT1* appears to be a regulator of metabolism, with a role in catalysing the first step in degradation of branched chain amino acids and contributing to glutamate production.[12–14] Dysfunctional regulation of *BCAT1* and *IKZF1* has been reported for several solid cancer types including CRC,[15] nasopharyngeal carcinoma,[16] epithelial ovarian cancer,[17] and lung cancer.[18]

Methylation-based detection of circulating tumour DNA (ctDNA) has emerged as promising analyte for non-invasive detection of many solid adenocarcinomas, including CRC.[19] We have recently shown that detection of ctDNA by assaying blood for methylated *BCAT1* and *IKZF1* DNA is sensitive and specific for detection of primary and recurrent CRC.[20–23] There is little research on the epigenetic regulation of *BCAT1* and *IKZF1* and effect on gene expression in tissue samples. This study aimed to assess association between the levels of DNA methylation and messenger RNA (mRNA) expression of *BCAT1* and *IKZF1* in cancer and adjacent non-neoplastic tissues of patients with CRC.

Materials and Methods

Study overview

This was a retrospective study assessing methylation and gene expression of *BCAT1* and *IKZF1* in colorectal tissue samples collected from patients undergoing primary surgery for CRC. The study was approved by the Southern Adelaide Clinical Human Research Ethics Committee and registered in the Australian and New Zealand Clinical Trials Registry (trial registration number 12611000318987). All patients gave written informed consent prior to any samples being collected.

Population

Patients aged between 18 and 85 years, who had been diagnosed with invasive CRC and had tissue samples banked in the Flinders Tumour Bank (Bedford Park, SA, Australia), were randomly selected for analysis in this study (n = 36). Tissue

Table 1. Oligonucleotide sequences used for gene expression analysis.

GENE	PRIMER	SEQUENCE (5′ TO 3′)	AMPLICON LENGTH (BP)	GENOMIC POSITION
HPRT1	Forward	TGACACTGGCAAAACAATGCA	94	Sixth exon
	Reverse	GGTCCTTTTCACCAGCAAGCT		
IKZF1	Forward	GCAAAGCTCCAAGAGTGACAGA	80	Second/third exon
	Reverse	AGGCACGCCCATTCTCTTC		
BCAT1	Forward	GGTCCCATATTCAACATCTGC	197	Fourth/fifth exon
	Reverse	CACCTTTCCAGGCTCTTACA		

samples were excluded from analysis if the patient had received chemotherapy or radiation treatment prior to resection. Demographic information and tumour characteristics (including stage, tumour depth of invasion, nodal involvement, presence of metastasis, and location) were obtained from the histopathology reports. Location of tumour was divided into colon and rectum. Cancers were staged according to AJCC Cancer Staging Seventh Edition.[24]

Collection of tissue samples

Tissue samples were collected from a fleshy non-necrotic part on the luminal surface of the resected cancer and from non-neoplastic tissue at least 5 mm proximal or distal to the tumour in patients undergoing surgical resection at either Repatriation General Hospital (Daw Park, SA, Australia) or Flinders Medical Centre (Bedford Park, SA, Australia). The samples were stored in RNAlater (Qiagen, Hilden, Germany) for at least 4 hours before they were transferred into new vials and stored at –80°C until further analysis.

Methylation testing

DNA was extracted from 15 mg tissue samples using the DNAeasy Blood & Tissue kit (Qiagen GmbH) according to the manufacturer's instructions. DNA concentrations were estimated by measuring the absorbance at 260 nm (NanoDrop 8000 UV-Vis Spectrophotometer; Thermo Fisher Scientific, Victoria, Australia). A total of 25 ng of DNA in 20-µL distilled water was bisulphite converted and purified using the EpiTect Fast Bisulfite Conversion Kit (Qiagen). A duplex polymerase chain reaction (PCR) assay targeting methylated regions residing in the promoters of *BCAT1* (promoter 1) and *IKZF1* as previously described[20–23] was performed on a Rotor-Gene Q instrument (Qiagen) in a final PCR volume of 15 µL (50 cycles: 94°C, 60 seconds: 60°C, 60 seconds). A bisulphite-conversion specific *ACTB* PCR assay was performed separately as the control assay.[20–23] The level of methylation was expressed as mass of methylated *BCAT1* or *IKZF1* DNA as a percentage of the total amount of analysed DNA (*ACTB*).

mRNA testing

Total RNA was extracted from approximately 30 mg cancer and non-neoplastic tissues using a TRIzol Plus RNA Purification Kit (Life Technologies, Mulgrave, Australia) as per manufacturer's recommendation (one tissue sample was not available for analysis). The concentration of RNA was estimated by measuring the absorbance at 260 nm (NanoDrop 8000 UV-Vis Spectrophotometer). For complementary DNA synthesis, 1 µg of RNA diluted in nuclease-free water was reverse-transcribed using a QuantiNova Reverse Transcription Kit (Qiagen). The reaction was performed at 25°C for 3 minutes, followed by 10 minutes at 45°C, whereafter the reaction was terminated by heating at 85°C for 5 minutes. The mRNA levels of *BCAT1, IKZF1*, and *HPRT1* (reference gene) were assessed in triplicate using a QuantiNova SYBR Green PCR Kit (Qiagen) and real-time PCR (Rotor-Gene Q instrument). Primers for the amplification of *BCAT1* were designed by Ensembl database (http://asia.ensembl.org/index.html), whereas *IKZF1* and *HPRT1* primers sets were obtained from published papers (Table 1).[25,26] Normalization was performed using *Q-Gene* application,[27] with the expression of *BCAT1* and *IKZF1* relative to *HPRT1* expressed as the mean normalized expression.

Statistical analysis

Statistical analyses were performed using GraphPad Prism, version 6.05 (GraphPad Software, Inc.). Normality of the data was tested with D'Agostino-Pearson normality test. Mann-Whitney and Kruskal-Wallis tests were used for group comparisons. The Spearman rank correlation coefficient was used for testing strength of correlations. χ^2 testing was used for proportion comparisons of samples with significant methylation (≥5%) between groups, with the Yates correction used when sample size was less than 5. Reported *P* values are 2-tailed and values less than .05 were considered statistically significant.

Results

The clinicopathological profiles of the patients enrolled in the study are shown in Table 2 and Supplementary Table 1. Median

Table 2. Comparison of clinicopathological parameters and levels of methylated *BCAT1* and *IKZF1* in cancer tissue.

| CLINICAL PARAMETERS	N=36	MEDIAN METHYLATED BCAT1 (25TH-75TH PERCENTILES)	P VALUE	MEDIAN METHYLATED IKZF1 (25TH-75TH PERCENTILES)	P VALUE
Gender					
Male	17	35.0 (13.4-53.3)		27.5 (0.8-87.3)	
Female	19	51.1 (21.1-77.5)	.311	62.0 (36.3-99.9)	.323
Age, y					
≤65	22	35.8 (13.4-75.7)		61.3 (7.5-109.7)	
>65	14	44.9 (30.9-64.7)	.804	47.8 (6.3-78.3)	.493
Location					
Colon	25	51.1 (25.2-81.9)		60.5 (25.5-83.6)	
Rectum	11	30.6 (12.1-47.2)	.106	45.6 (0-124.8)	.912
Tumour invasion depth (T stage)					
T1	4	56.9 (30.8-87.13)		44.0 (3.3-92.9)	
T2	7	51.1 (26.8-89.45)		62.5(30.3-92.4)	
T3	16	40.9 (22.3-68.9)		53.25 (30.0-105.3)	
T4	9	34.2 (7.8-47.6)	.505	27.5 (0- 62.0)	.612
Lymph node involvement (N stage)					
Yes	15	35.0 (13.8-62.9)		41.2 (0.4-68.8)	
No	21	46.8 (24.9-76.6)	.361	60.5 (12.1-116.1)	.402
Distant metastasis (M stage)					
Yes	5	35.0 (0.8-36.6)		36.7 (27.5-62.0)	
No	31	46.8 (21.0-74.9)	.245	56.5 (2.2-94.5)	.989
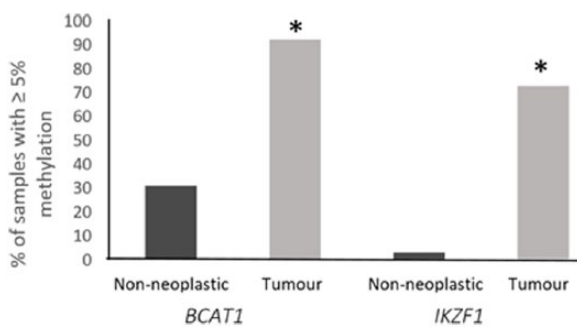
Figure 1. The proportions of tumour and non-neoplastic tissues with methylated *BCAT1* and *IKZF1* (n=36). Samples were considered to have significant levels of methylation when levels were ≥5%. *$P < .05$ compared with non-neoplastic results.

age was 63.4 years (25th-75th percentiles: 52.6-72.4 years). About 82% of patients presented with local disease (stage I = 8, stage II = 13, stage III = 10) at diagnosis, whereas the remainder had metastases (stage IV = 5).

Significantly higher levels of methylation were observed for *BCAT1* and *IKZF1* in CRC tissue compared with adjacent non-neoplastic tissue. Using a % methylation threshold of 5%, *BCAT1* was methylated in 91.7% cancers (33/36) and 30.6% of non-neoplastic tissues samples (11/36; $P < .001$; Figure 1). Similarly, *IKZF1* was methylated in 72.2% (26/36) of cancers and 2.8% (1/36) of non-neoplastic tissues ($P < .001$; Figure 1). About 97.2% (35/36) of all cancer tissues samples had significant methylation in either *BCAT1* or *IKZF1*. There was a significant positive correlation between the percentage methylation of *BCAT1* and *IKZF1* of cancer tissues ($R = .486$; $P = .003$).

No significant associations of *BCAT1* or *IKZF1* methylation levels with patient age or sex or cancer location were observed ($P > .05$; Table 2). The relationship between CRC stage and levels of methylated *BCAT1* and *IKZF1* is shown in Figure 2. Although stage IV cancers appeared to have lower methylation levels for both *BCAT1* and *IKZF1*, there were no statistical differences between the stages (% methylation [median], stage I to IV: *BCAT1*, 47.0%, 46.8%, 40.9%, and 35%; $P = .650$: *IKZF1* = 61.5%, 56.5%, 43.4%, and 36.7%; $P = .820$). There were also no correlations between levels of methylated *BCAT1* and *IKZF1* in cancer tissues and the depth
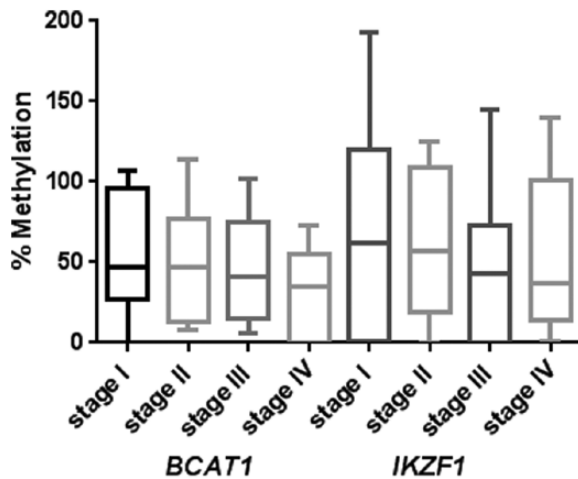
Figure 2. CRC stage-related methylation levels of *BCAT1* and *IKZF1*. Levels of methylated *BCAT1* and *IKZF1* in relation to CRC stage (TNM staging). Different CRC stages, stage I (n = 8), II (n = 12), III (n = 11), and IV (n = 5), are plotted on the *x*-axis, and the percentage of methylation of each gene (*BCAT1* and *IKZF1*) is plotted on the *y*-axis. Data are expressed as median, 25th and 75th percentiles within the box, with bars showing the minimum to maximum. CRC indicates colorectal cancer.
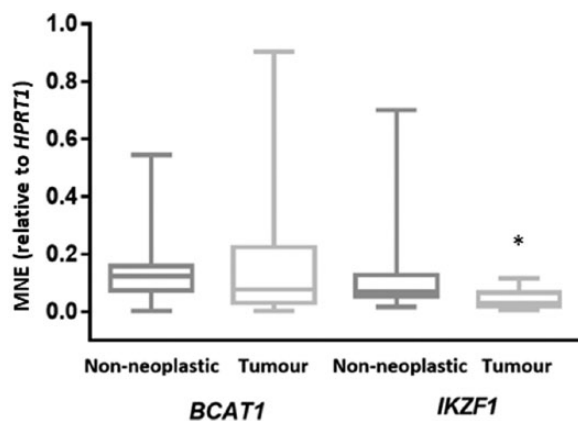

Figure 3. Relative messenger RNA expression of *BCAT1* and *IKZF1* in colorectal tumour and adjacent non-neoplastic tissues (n = 36). The expression results are normalized to the levels of *HPRT1* expression in corresponding samples and expressed as mean normalized expression. Data are expressed as median, 25th and 75th percentiles within the box, with bars showing the minimum to maximum. *$P < .05$ compared with non-neoplastic results.

of invasion (T stage), lymph node invasiveness (N stage), or presence of metastases (M stage) (Table 2).

To determine whether methylation of *BCAT1* and *IKZF1* extended beyond the tumour tissue, methylation in the tissue collected close to the tumour (distance ≤50 mm of tumour: n = 15, median = 25 mm) was compared with that of tissue sampled distant to the tumour (distance >50 mm of tumour: n = 20, median = 93 mm). One sample was excluded from analysis due to position not being recorded. There were no significant differences in the median methylation of either gene in the non-neoplastic tissue collected close to the tumour compared with further away (≤50 mm of tumour, median % methylation: *BCAT1* = 3.2%, *IKZF1* = 0%; >50 mm of tumour, median %

methylation: *BCAT1* = 3.0% and *IKZF1* = 0%; $P = .980$ and .853, respectively).

The levels of *BCAT1* and *IKZF1* mRNA expression in cancer tissues and adjacent non-neoplastic tissue samples are shown in Figure 3. The levels of *IKZF1* mRNA in cancers (relative expression, median = 0.030) were significantly lower than that of the adjacent non-neoplastic tissues (median = 0.070; $P < .001$). The *BCAT1* mRNA levels were not significantly different in cancers (median = 0.077) and adjacent non-neoplastic tissues (median = 0.125; $P = .799$).

When comparing the levels of methylation and mRNA expression of *BCAT1* and *IKZF1* in cancers and the matching non-neoplastic tissues, a significant correlation was observed for *IKZF1* ($R = -.482$; $P < .001$). There were 8 cancer tissues without methylation in *IKZF1*, but their levels of *IKZF1* mRNA were not significantly different from those tumours with high *IKZF1* methylation levels (median relative gene expression of 0.033 and 0.029, respectively, $P = .685$); however, sample size is small for cases with no methylation. No significant correlation was seen between levels of *BCAT1* methylation and mRNA expression ($R = -.017$; $P = .885$).

Discussion

The role of aberrant methylation in human tumorigenesis has been studied intensively,[28] with hypermethylation of CpG islands, often embedding promoter regions, associated with cancer development and progression.[7] This study evaluated the *BCAT1* and *IKZF1* methylation and mRNA levels in cancer and matched non-neoplastic tissues of patients with CRC.

The methylation assessment method used in this study has previously been used for detection of methylated *BCAT1* and *IKZF1* in plasma samples.[22] Assessment in tissue samples showed that cancer tissues contained significantly higher levels of methylated *BCAT1* and *IKZF1* DNA compared with adjacent non-neoplastic tissues. These results, along with our previous findings that DNA extracted from plasma samples from patients with CRC is highly methylated for *BCAT1* and *IKZF1* compared with healthy controls,[6,20] suggest that the presence of methylated *BCAT1* and *IKZF1* DNA in the blood likely comes directly from the cancer, rather than just being a general physiological response of the body to the presence of neoplastic lesion.

Tumour tissue levels of methylated *BCAT1* and *IKZF1* were not significantly different between the different stages of CRC assessed suggesting that hypermethylation of these 2 genes in CRC is an early event in oncogenesis. In support of this, *BCAT1* methylation has been proposed as an early event during nasopharyngeal carcinoma pathogenesis,[16] and Kisiel et al[29] showed that the hypermethylation of *IKZF1* associated with pancreatic cancer does not depend on the stage of the disease. Moreover, the lack of correlations between levels of methylated *BCAT1* and *IKZF1* and the depth of invasion (T stage), lymph node invasiveness (N stage), and the presence of metastases (M stage) observed in this study further supports

the observations that epigenetic abnormalities of both *BCAT1* and *IKZF1* are independent of stage and probably early events. A limitation, however, of this study was that the analysed biopsy samples were taken only from the luminal surface of the cancer. It is possible that differences in methylation levels could be found throughout the cancer. A previous study that analysed 3 different regions of CRC revealed the presence of heterogenic DNA methylation patterns within each cancer, with the greatest methylation found in the central bulk of the cancer.[30] Despite this, in this study, we found that 97% of tumour tissues contained significant levels of methylated *BCAT1* and/or *IKZF1*. A positive correlation was also observed between levels of methylated *BCAT1* and methylated *IKZF1* in CRC tissues. This has also been reported for plasma samples of patients with CRC.[20]

DNA methylation profiles have previously been found to vary between the colon and the rectal cancers,[31] as well as being associated with clinical parameters including sex[32] and age.[33] In this study, we observed that patient demographics including age and sex, as well as cancer site, had no effect on the levels of methylated *BCAT1* and *IKZF1* in cancer and non-neoplastic tissues. In agreement, one other study that assessed levels of methylated *IKZF1* in CRC tissue found no correlation with age, sex, or cancer location.[15] In our study, non-neoplastic tissues had little to no methylation. Furthermore, levels of methylation were not associated with any patient feature. We can therefore conclude that changes in the level of methylated *BCAT1* and *IKZF1* reported in this study are related to oncogenic progression rather than effects of age, sex, and/or site.

In this study, a significant downregulation of *IKZF1* gene expression was observed in cancer tissues compared with adjacent non-neoplastic tissue. The sample size was too small to confirm whether the downregulation of expression is a consequence of hypermethylation, and therefore demethylation experiments in CRC cell lines are warranted for further elucidation. Some cell line studies have reported hypermethylation-induced silencing of *IKZF1* in different cancer types including lung cancer[18] and acute lymphoblastic leukemia.[34] The functional roles of *IKZF1* in CRC progression are not understood, but it is possible that *IKZF1* could function as tumour suppressor gene during CRC development. This suggestion is strongly supported by the findings of Javierre et al[15] who reported a significant reduction in colony formation and cell viability of HCT-116 following ectopic expression of *IKZF1*. The *IKZF1* gene resides in a long-range epigenetic silencing region, which also harbours genes that have roles in limiting growth and differentiation (*PTPN6* and *MEIS2*) and the long-range silencing of this region may contribute to tumorogenesis.[15]

Despite the significant levels of methylation measured in the *BCAT1* promoter 1 in cancer tissues, this was not reflected in the mRNA level. This may be due to the *BCAT1* mRNA PCR assay detecting more than one mRNA transcript. A limitation of this study was that the *BCAT1* mRNA assay used was not mRNA transcript specific. Therefore, this may have led to an incorrect estimation of *BCAT1* mRNA levels. In addition, the expression of the different *BCAT1* transcript isoforms (T1, T4, and T6), at least in gliomas,[35] is controlled by 2 different promoter regions (T1 expression is regulated by promoter 1, whereas T4 and T6 expressions are regulated by promoter 2). The transcripts differ only in their first exons but have been shown to correspond to a single protein.[35] This study only assessed methylation in promoter 1. The study in gliomas showed that hypermethylation of promoter 1 caused upregulation of gene expression (T1 isoform), whereas hypermethylation of promoter 2 was associated with downregulation of expression (T4 and T6 isoforms).[35] Another study reported an association between *BCAT1* methylation and gene silencing in lung cancer[36]; however, this study did not provide details on transcripts assessed. A recent study in acute myeloid leukaemia[37] showed that overexpression of *BCAT1* was associated with DNA hypermethylation and poor prognosis, whereas in CRC, dysfunctional *BCAT1* expression was associated with distant metastasis and a predictor of short disease-free survival.[38] The dysfunctional effect of *BCAT1* appears to vary between cancer types,[39] but loss of regulation appears to be in cancer proliferation, migration, and invasion. Studies are needed to further understand the role of the association of methylation of the different promoters with *BCAT1* gene expression in CRC tissue.

Conclusions

This study has shown that colorectal tumours, but not the adjacent non-neoplastic tissue, are highly methylated for *BCAT1* and *IKZF1* at all stages of CRC. A positive correlation was observed between hypermethylated *IKZF1* and silencing of gene activity. Further studies are therefore warranted to elucidate the role of *IKZF1* in CRC development.

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Author Contributions

MJ, GPY, and ELS planned the study design. MJ performed the experimental work and assessment of cancer clinical features. MJ, SKP, and ELS analysed and interpreted the data. All authors read and approved the final manuscript.

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