The effects of neoadjuvant therapy on the methylation levels and gene expression of *BCAT1* and *IKZF1* in colorectal tumour and para-tumour tissues

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Abstract

Globally, colorectal cancer (CRC) is the second most commonly diagnosed cancer in females and the third in males, with 1.4 million new cases estimated to have occurred in 2012. This disease arises as a consequence of several genetic and epigenetic modifications, including DNA methylation. Blood tests are being developed to detect these methylation changes for CRC screening. One such test is based on the presence of circulating hypermethylated *BCAT1* and *IKZF1*, but little work has been done to measure methylation levels in the colorectal tumour and non-tumour tissue, or to determine the impact that methylation may have on the gene expression levels. In addition, it is not known how treatment (radiation/chemotherapy) of the tumour may affect methylation and gene expression. The aim of this study was to assess levels of methylation and gene expression of *BCAT1* and *IKZF1* in tumour and para-tumour tissues of CRC patients and to determine the effect of treatment on these levels. Tumour and adjacent non-tumour (para-tumour) tissues were collected at surgical resection of 50 patients diagnosed with different stages of CRC. Of these patients, 14 were subjected to treatment prior to surgical resection. DNA was extracted, bisulfite converted and levels of methylated *BCAT1* and *IKZF1* were evaluated via quantitative PCR and expressed as a percentage of β-actin levels. The relative gene expression levels of *BCAT1* and *IKZF1* were assessed through measuring mRNA levels by first extracting RNA, preparing cDNA and then performing real-time PCR to measure expression relative to a reference gene (*HPRT1*). Comparisons between the different groups were performed with a Mann Whitney U-test (p values <0.05 considered significant). High levels of methylated *BCAT1* and *IKZF1* were observed in tumour tissues (39.8%; 53.3%, respectively) compared with para-tumour tissues (3.2%; 0%, $p<0.001$). The increased *IKZF1* methylation level was associated with a decrease in gene expression levels, with the tumours levels (0.030) significantly lower than the

expression in para-tumour tissues (0.069; $p<0.0001$). No differences were observed in *BCAT1* gene expression between tumour (0.077) and para-tumour (0.1247; $p=0.7993$). Compared to non-treated tissue, treatment reduced methylated *BCAT1* and *IKZF1* in tumour tissue (0.5%; 0.5%, $p<0.01$) without significantly affecting levels in para-tumour tissue (7.7%; 0.0%, $p>0.05$). The reduction in tumour methylation was accompanied by an increase in both *BCAT1* (0.35) and *IKZF1* (0.10) gene expression compared to non-treated levels ($p<0.05$). The levels of methylated *BCAT1* and *IKZF1* within the tumour was associated with the response to therapy, with significantly lower levels in tumours that had shrunk to 50% or less of the original size (both = 0%), compared to tumours with less size reduction (6.1% and 5.3%, $p<0.05$). Colorectal tumours, but not the adjacent non-tumour tissue, are highly methylated for *BCAT1* and *IKZF1*. Methylation significantly reduced gene expression levels of *IKZF1*, but not *BCAT1*. Therapy reduced methylated *BCAT1* and *IKZF1* levels of the tumour and increased expression of *BCAT1* and *IKZF1*. Levels of methylation may be able to be used as a marker for treatment success prior to surgical intervention.

Declaration

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis.

Signed

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Abbreviations

ACTB	Actin beta
AJCC	American Joint Committee on Cancer
BCAA	Branched chain amino acids
BCAT1	Branched chain amino acid transaminase 1
cDNA	Complimentary DNA
CG content	Cytosine and guanine content
CIMP	CpG island methylation phenotype
CpG	Cytosine-phosphate-guanine
CRC	Colorectal cancer
Ct	Threshold cycle
DNA	Deoxyribonucleic acid
DNTM	DNA methyltransferase
E	Efficiency
gDNA	Genomic deoxyribonucleic acid
Gy	Gray
HDAC	Histone deacetylase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
IKZF1	IKAROS family zinc finger 1
IR	Ionizing radiation
miRNA	Micro ribonucleic acid
ml, µl	Milliliter, microliter
MNE	Mean Normalized Expression
ng, mg	Nanogram, milligrams
nm	Nanometer
nmol	Nanomole
NuRD	Nucleosome remodeling and deacetylation complex

PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SEER	The Surveillance, Epidemiology, and End Results database
TNM	Tumour, nodes, metastasis
USPSTF	The US Preventive Services Task Force
xg	Times gravity

Chapter 1. Introduction

1.1 Study overview

Colorectal cancer (CRC) is the third major fatal cancer worldwide. Development of this disease requires several genetic and epigenetic modifications. While many studies are working to reduce death rate from CRC through the development of new treatments and early diagnostic methods, there has been very little work into developing non-invasive techniques to monitor the effectiveness of neoadjuvant treatments (radiotherapy and chemotherapy) and patients' response. Blood tests to detect the methylation changes associated with CRC have mainly been developed for early diagnosis. One such test is based on the presence of circulating hypermethylated *BCAT1* and *IKZF1*. Alterations to *BCAT1* and *IKZF1* have been reported in other cancers but little work has been done on their roles in CRC within tissue samples. Analysis of the levels of methylation and gene expression of *BCAT1* and *IKZF1* in tumour and para-tumour tissues and the determination of how these levels are related to patients' pathological parameters could represent a step towards understanding *BCAT1* and *IKZF1* functions in CRC. The aim of this study was to assess levels of methylation and gene expression of *BCAT1* and *IKZF1* in tumour and para-tumour tissues of CRC patients and to determine the effect of treatment on these levels. This study could further confirm the use of methylation levels of *BCAT1* and *IKZF1* as a non-invasive screening tool for CRC, as well as determining if the methylation levels of these biomarkers could also be applied as prognostic biomarkers to predict patients' response to treatments. The following sections will give general introduction to cancer, then explain the incidence of colorectal cancer, how epigenetic changes contribute to the development of the disease, and what techniques are available to aid in detection and treatment.

1.2 Cancer incidence and mortality

An increasing cancer burden through the next decades is expected worldwide especially in the countries where the low and middle-income levels are prevalent. This increase in cancer burden is predicted to escalate dramatically and reach more than twenty million new cases at the beginning of 2025 annually (Stewart & Wild 2014). In the United States, the 2013 annual estimation of cancer showed that one out of each four deaths is due to cancer indicating that cancer is a major health problem not only in the developing countries, but also the developed countries (Siegel, Miller & Jemal 2015). According to GLOBOCAN estimates in 2012, globally there were approximately 14.1 million and 8.2 million new cancer cases and cancer deaths respectively in both males and females caused by 27 major cancers, where 1.82 million, 1.67 million and 1.36 million cases were diagnosed with lung, breast, and CRC respectively (Ferlay et al. 2014). Moreover, it was revealed that in 2012 the highest rate of mortality in men was caused by lung cancer (~1.25 million deaths) while breast cancer represented the major cause of death in women (~522,000 deaths). In the same year, 746,000 men and 614,000 women were diagnosed with CRC (Ferlay et al. 2015). CRC which was ranked as the third most common type of cancer in men and the second most common type of cancer in women was responsible for 694,000 deaths in both genders (Ferlay et al. 2015). Although morbidity and mortality from digestive system cancers especially CRC are still high (Siegel, Naishadham & Jemal 2013), over the past few years, the screening techniques that have been developed and allow for the early diagnosis and removal of pre-cancerous polyps, and improved treatments, have reduced the death and incidence rates up to 3.0% in both males and females (Horner et al. 2009; Siegel, Naishadham & Jemal 2013).

1.3 Differences between cancer and non-cancer cells

In general, cancer is the consequence of a defect of the essential homeostatic mechanisms that are required to control and maintain the equilibrium within and between cells in an organism (Paul 1962). Cancer can be categorized according to the type of tissue at which cancer initiates or according to original site in the body in which cancer first established. Hundreds of kinds of cancer have been identified; however, from a histological point of view, all of them are classified into six main groups: carcinoma, sarcoma, myeloma, leukemia, lymphoma and mixed types (National Cancer Institute 2015). Previously, many studies have investigated the differences between cancerous and non-cancerous cells, highlighting modifications adopted by cancer cells in order to live and thrive in conditions considered unfavorable or even toxic to other normal cells. In the following sections the focus will be on the genetic and the epigenetic modifications occurring frequently during cancer development.

1.3.1 Genetic alterations

Genetic alterations in cancer cells differ among the various types, stages and tissues, and they range from single nucleotide mutation to overall chromosomal aneuploidy. Accumulation of DNA changes is known to be a critical part of cancer etiology that is responsible for uncontrolled gene expression profile and disorganized signaling system (Hahn & Weinberg 2002; Sadikovic et al. 2008). The phenotypic consequences of genetic alterations of cancer cells during tumorigenesis, including deregulated transcription and translation, allow researchers to categorize cancer genes into oncogenes and tumour suppressor genes. While oncogenes represent genes that under certain circumstances can promote cancer, tumour suppressor genes can protect cells from being transformed into cancer by either inducing cell survival or cell death pathways (Hanahan & Weinberg 2000). Several genetic alterations are required for full development of cancer, and these

changes may include both activation of oncogenes and inactivation of tumour suppressor genes where cumulative responses of such alterations might be evident in tumours characterized by high rates of genomic instability (Sadikovic et al. 2008).

1.3.1.1 Disruption of tumour suppressor genes

The main kinds of alterations which lead to inactivation of tumour suppressor genes are genetic mutations, deletions and allelic losses (Sadikovic et al. 2008). In general, intragenic mutation is a type of mutation influencing genes that are either responsible for maintaining genomic integrity or play a role in tumour suppressor pathways (Efeyan & Serrano 2007; MacPherson & Dyer 2007). In humans, *p53* is considered one of most frequently mutated tumour suppressor genes and its alteration mediates the pathogenesis of several tumours such as brain and breast tumours. Glioblastoma which is a brain tumour is an example where a mutation that inactivates *p53* occurs as an early event during glioblastoma development (Fuchs & Pritchard 2002; Ohgaki & Kleihues 2007; Papachristou & Papavassiliou 2007). Other examples of mutated tumour suppressor genes are *APC* in colon, thyroid, intestine and stomach cancers (Arnold et al. 2004; Mori et al. 1992); and *BRCA1* and *BRCA2* in breast and ovarian cancer (Bianco et al. 2000; Tseng et al. 1997). In addition to inactivation mutations which are commonly single nucleotide alterations, inactivation of tumour suppressor genes can happen due to deletions of large pieces of chromosomes, or even loss of entire chromosomes. The effects of deletions and allelic loss can be seen in retinoblastoma with *INK4* genes which are tumour suppressor genes that act as regulators of cell cycle controlling G1 to S transition (Giacinti & Giordano 2006; Wei et al. 1999). Another tumour suppressor gene affected by chromosomal deletion is *PTEN* which is required for modulation of PI3K, phosphatidylinositol-3-kinase, pathway and activation of AKT, a regulatory protein kinase (Yoshimoto et al. 2007).

1.3.1.2 Activation of oncogenes

As a result of mutations, chromosome translocations and gene amplifications, activation of oncogenes might occur. When oncogenes are activated, the structure of oncogene-encoded proteins is modified in a way that causes activation of their transforming function. RAS oncogene family is a classic example of an oncogene which when mutated, produces proteins with a continuously active state which in turn cause constant cell proliferation (Bedford & Van Helden 1987; Rodenhuis 1992). Activation of oncogenes can also be induced by chromosomal translocations where rearrangements in chromosomes can produce genes that encode oncogenic functional proteins. For example, the fusion of *ABL* oncogene to *BCR* gene forms an oncogenic protein called BCR/ABL which is characterized by constant tyrosine kinase activity and commonly happens in chronic myelogenous leukemia (Groffen et al. 1984; Shtivelman et al. 1985). Likewise, chromosomal rearrangements can activate transcription factors stimulating overexpression of oncogenes (Bonin et al. 1993) such as *EWS* gene that can fuse with a wide range of genes to produced RNA-binding EWS protein which interacts with DNA-binding-domain and enhances oncogene overexpression (Arvand & Denny 2001). In addition, genomic amplification can also cause activation of oncogenes where these modifications usually take place through cancer progression. Genomic amplification can be observed in several oncogene families such as MYC, CCND, and FOS where the amplified piece of DNA that includes several genes involving oncogenes are overexpressed to form oncogenic functional proteins (Bhattacharya et al. 2005).

1.3.2 Epigenetic alterations

In 1940, the term ‘epigenetic’ was used for the first time to describe the causal analysis aiming to identify the developmental processes (Slack 2002). Currently, epigenetic is used to describe the hereditary alterations of gene expression that occur without influence on the sequence of the gene

(Bernstein, Meissner & Lander 2007). These non-genetic alterations are transmitted as enzymatically induced and covalently attached chemical changes of cytosine bases, where the spatial and temporal allocation of such modifications may play essential roles in controlling gene expression, possibly by altering the level of DNA packaging that makes genetic materials more or less accessible. Epigenetic alterations include several mechanisms such as DNA methylation of cytosine bases, acetylation, and methylation of histones, chromatin remodelling and small and non-coding RNAs (Kim, Samaranayake & Pradhan 2009; Sadikovic et al. 2008).

1.3.2.1 DNA methylation

DNA methylation is a well-characterized inherited epigenetic change that participates in regulation of DNA accessibility and chromatin compaction, allelic imprinting, X chromosome activity and DNA silencing (Kim, Samaranayake & Pradhan 2009). DNA methylation refers to the process of covalently adding a methyl group to a fifth carbon found in the cytosine ring, and consequently 5-methyl-cytosine is produced. The majority of cytosine methylation occurs within a CpG dinucleotide, a linear sequence of cytosine and guanidine, which is part of a region of 200 bp long and more than 50% CG content called CpG islands (Jaenisch & Bird 2003). Cytosine methylation is mediated by enzymes called DNA (cytosine-5) methyltransferases (DNMTs) which can by classified into maintenance DNMTs and de novo DNMTs. The maintenance DNMTs act on the hemi-methylated DNA to maintain the methylation patterns during cell division whereas de novo DNMTs act during cell development to initiate methylation patterns on certain locations (Holliday & Pugh 1996; Riggs 1975).

Regulation of gene expression by aberrant DNA methylation can cause initiation and development of various diseases such as cancer. Abnormal DNA methylation profiles in cancer cells can be seen in different forms including promoter hypermethylation and genomic hypomethylation

(Sadikovic et al. 2008). One of the common features of the human genome is the abundance of CpG dinucleotides in promoter regions of almost 50% of genes. Usually CpG enriched regions of promoters are not methylated so genes can be expressed normally. However, hypermethylation of CpG islands, frequently occur during cancer, can cause transcription inactivation of several important genes associated with apoptosis, cell cycle control, angiogenesis and DNA repair (Antequera & Bird 1993; Das & Singal 2004; Momparler & Bovenzi 2000). The role of hypermethylation in cancer initiation is supported by genome wide studies of cancer epigenomes. These studies indicate that about 10% of CpGs, thousands of promoters, are abnormally methylated in various cancers (Costello et al. 2000; Pfeifer & Rauch 2009).

Another DNA methylation abnormality is overall hypomethylation of the genome (Ehrlich 2002). It is believed that genomic hypomethylation can induce cancer development by either activation of oncogenes such as *c-MYC* (Tsukamoto et al. 1992) or by promoting instability of chromosomes (Tuck-Muller et al. 2000). This defect can be observed in several types of cancer including hepatocellular (Lin et al. 2001), cervical (Kim et al. 1994), colorectal and prostate cancers (Bedford & Van Helden 1987; Wahlfors et al. 1992).

DNA methylation can cooperatively interact with other proteins to regulate gene expression such as the interaction with the family of proteins called methyl-binding proteins. The human genome encodes for several methyl-binding proteins such as MBD1, MBD2, ZBTB4, ZBTB38, UHFR1 and UHFR2 (Kirmizis et al. 2004; Van Engeland et al. 2011). These proteins are characterized by their strong affinity for methylated DNA and their ability to regulate chromatins structures by inducing histone modification and chromatin condensation. Binding of methyl-binding proteins with methylated DNA triggers a series of events that induces compacted chromatin status and subsequently block the accessibility of transcription factors to promoter sequences (Bird 2002).

1.4 Colorectal cancer

Cancer of the colon and rectum is an extensively studied disease that has been researched for the genetic and epigenetic causes. Worldwide, bowel cancer is the third most frequently occurring cancer, where in 2008 a total of 1.2 million new CRC cases were recorded (Boyle & Leon 2002). The geographical distribution of CRC varies among countries, with high incidence rates in USA, Europe, Australia and New Zealand, and lower incidence rates in Africa and Asia (Boyle & Leon 2002). The mortality of CRC is about half of the incidence. In 2008 there were about 600,000 deaths from CRC worldwide, making CRC the third leading cause of death of all cancers. However, mortality rates are reducing in the developed countries as a result of early diagnosis and improved treatments (Siegel, DeSantis & Jemal 2014).

1.4.1 CRC staging

A classification scheme that can be used to determine the clinical staging of cancer at various anatomic sites, is an important tool that enables physicians to choose the appropriate treatment regimen and provide basis for prediction of cancer survival rate. Efforts for developing a uniform and universally accepted cancer classification system were started early in the 19th century (O'Connell, Maggard & Ko 2004). Since then several editions of cancer staging manuals have been published, where the formal TNM staging system has been applied as a standard method for identifying the extent of most types of cancer at a particular time. Initially, TNM staging system was proposed by the American Joint Committee on Cancer (AJCC). This shorthand system is based on a combination of three components: size (depth) of the tumour (T), the involvement of regional lymph node (N), and the existence of distant metastases (M) (O'Connell, Maggard & Ko 2004).

Size and depth of invasion of primary tumour are described as either TX: primary tumour cannot be assessed, T0: no evidence of primary tumour, Tis: carcinoma *in situ*, T1: tumour has extended through the submucosa of intestine, T2: tumour invades muscularis propria, T3: tumour has grown through the serosa or T4: tumour has come in contact with nearby structures or organs. In contrast, the involvement of regional lymph nodes is represented by NX: lymph nodes cannot be assessed, N0: no lymph node is invaded, N1: tumour spreads into 1 to 3 lymph nodes or N2: tumour metastasis in 4 or more lymph nodes. The presence and absence of metastasis is displayed by MX: metastasis cannot be assessed, M0: no distant metastasis or M1: the present of distant metastasis (O'Connell, Maggard & Ko 2004).

By applying TNM staging system, colorectal tumours can be categorized into either stage 0, I, II, III or IV (Table 1). The earliest stage of CRC is stage 0 in which an abnormal growth of epithelial cells in the mucosa of the colon or the rectum can be found. Stage I is the earliest stage of invasive cancer, while stage IV is the latest stage in which tumour has spread to distant sites such as liver and lungs (O'Connell, Maggard & Ko 2004).

Table 1. Summary of anatomic TNM staging of CRC (O'Connell, Maggard & Ko 2004).

Stage	Size and depth of tumour (T)	Involvement of regional lymph node (N)	Existence of distant metastases (M)
0	Tis	N0	M0
I	T1 or T2	N0	M0
IIA	T3	N0	M0
IIB	T4	N0	M0
IIC	T4	N0	M0
IIIA	T1-T2	N1	M0
IIIB	T3-T4	N1	M0
IIIC	Any T	N2	M0
IV	Any T	Any N	M1

1.4.2 Survival rate of CRC

In general, the survival rates of cancer are an indication of the proportion of people with a similar type and stage of cancer, who are likely to be alive for given period, usually 5 or 10 years, after being diagnosed with the disease (American Cancer Society 2016; Cancer Research UK 2015).

In CRC, the survival rates have improved significantly over the last few years. According to Bowel Cancer Australia, the 5-year survival rate of CRC increased to 66% between 2006 and 2010 after being 48% between 1982 and 1987 (Bowel Cancer Australia 2014).

According to the Surveillance, Epidemiology, and End Results database (SEER) of the National Cancer Institute, the survival rates of patients who have been diagnosed with various stages of either colon or rectal cancers between 2004 and 2010 are as follows. First, at the early stages of colon and rectal cancer (stage I), which have the highest curability, it was estimated that the 5-year survival rates were about 92% and 87% respectively. Second, slightly higher 5-year survival rates for people with either stage IIA or stage IIB of colon cancer were reported compared with the 5-year survival rates of people with either stage IIA or stage IIB of rectal cancer. For stages IIIA, IIIB and IIIC colon cancer, the 5-year survival rates were 84%, 71%, and 58% respectively while for stages IIIA, IIIB, and IIIC rectal cancer the 5-year survival rates were 89%, 69%, and 53% respectively. Finally, for the advanced cases of cancer, stage IV, with the lowest level of curability and poorer outlook caused by metastasis, the 5-year survival rates of colon and rectal cancers were lower than all the other stages (American Cancer Society 2016) (Table 2).

Table 2. The 5-year survival rate of colon and rectal cancer by stage of diagnosis (2004-2010) (American Cancer Society 2016).

Colon cancer staging	% 5-year survival rate	Rectal cancer staging	% 5-year survival rate
Stage I	92	Stage I	87
Stage IIA	87	Stage IIA	80
Stage IIB	63	Stage IIB	49
Stage IIIA	84	Stage IIIA	89
Stage IIIB	71	Stage IIIB	69
Stage IIIC	58	Stage IIIC	53
Stage IV	11	Stage IV	12

1.4.3 Genetic modifications associated with CRC

As mentioned before, CRC is considered the third most diagnosed type of cancer. Almost three-quarters of patients suffer from the sporadic form of CRC and the last quarter of patients have a family history of the disease. Only a small portion of about 4 - 7% of familial forms occur as a result of heritable mutations in dominant genes while the vast majority of them are due to the interaction between penetrant genes and environmental agents. Multiple genetic mutations in combination with acquired genomic instability are identified as fundamental cellular features required for CRC development (Tang et al. 2004). Most colorectal tumours develop through the adenoma-carcinoma sequence, with others through the serrated pathway (Al-Sohaily et al. 2012). The adenoma-carcinoma pathways involve multiple steps in which the normal colon epithelial cells develop into a benign polyp that eventually transform to a malignant tumour over a period of 10-15 years (Lao & Grady 2011). Mutation of the tumour suppressor gene adenomatous polyposis coli (*APC*) was found as one of the early steps during the malignant transformation. After that accumulation of several genomic mutations including *K-ras* mutation, *p53* deletion, *hMSH2* inactivation and *DCC* deletion occurs (Lao & Grady 2011). Three major molecular pathways of genomic instability are well-documented including chromosomal instability, microsatellite

instability, and CpG island methylator phenotype pathways (Armaghany et al. 2012; Jass 2007; Tang et al. 2004).

1.4.4 Methylation abnormalities associated with CRC

In general, both cancer-induced methylation abnormalities (hypermethylation and hypomethylation) have been observed during CRC development (Feinberg 2004; Van Rijnsoever et al. 2002). Hypermethylation which occurs at the CpG enriched promoter sequences or upstream genes regulation regions, is required for tumour suppressor gene silencing (Carethers & Jung 2015). In contrast, hypomethylation which is also termed body methylation because it takes place outside genetic promoter sequences is believed to be essential to stimulate the expression of oncogenes through transcriptional activation events (Hernandez-Blazquez et al. 2000).

The first discovery of epigenetic alterations in CRC goes back to 1982 where an entire loss of 5-methylcytosine content was shown in a sample of colon cancer compared to normal colonic mucosa tissue (Feinberg & Vogelstein 1983). Since that time, it has been shown that more genes in the genome of colon cancers are characterized by abnormal methylation patterns than by genetic mutations (Schuebel et al. 2007; Tsai & Baylin 2011).

In CRC, a specific set of genes has been found to be frequently methylated (Toyota et al. 1999). The presence of a cancer-distinct methylated set of genes suggests the existence of specific forces driving and controlling the process of gene methylation. For the abnormal DNA methylation in CRC, there may be more than one possible underlying force including the selective targeting which reflects the tendency of specific genes for methylation; the selective advantage that represents the clonal growth of cells containing genes with aberrant methylation patterns; or a mixture of both mechanisms (Gardiner-Garden & Frommer 1987). With regards to the pathways involved in selective targeting, two models can be used to explain the abnormality of gene methylation. First,

the overexpression, activation, and misdirection of DNMTs. Second, impairment of barrier elements that protect specific regions of DNA from being methylated.

Increased expression and activation of DNMTs has been proposed to cause abnormal DNA methylation in several cancers including CRC where the majority of DNMTs effects are focused at promoter sequences (Ibrahim et al. 2010; Issa et al. 1993). This proposal is supported by results of studies performed in cell lines and animal models which demonstrate that the increased expression of DNMT1 and DNMT3b can promote the occurrence of abnormal methylation within CpG islands in ways similar to that observed in CRC (McCabe, Lee & Vertino 2009; Nosho et al. 2009; Schmidt et al. 2007; Steine et al. 2011). However, results of a study in primary colon cancer indicate that there is no evidence for overexpression of DNMT (Ehrlich et al. 2006). Therefore, it has been suggested that the disorder could be misdirected activity of DNMT instead of the elevated activity. This suggestion is confirmed, in part, due to the ability of certain histone methyltransferases including EHMT2 and PRMT5 to direct the activity of DNMTs to specific locations. Accordingly, when these histone methyltransferases are disrupted, as seen in some cancers, DNMTs are directed to incorrect loci and mediate abnormal DNA methylation (Ohm et al. 2007; Schlesinger et al. 2007).

Another mechanism that has been postulated to be associated with abnormal methylation seen in promoter regions is the weakening of barrier elements. These elements are believed to be formed by assembling specific nucleotides sequences with associated proteins, and they serve as impediments that prevent the flow of incorrect signals including abnormal methylation signals, throughout the genome (Tsai & Baylin 2011). The phenomena of methylation signal diffusion from heavily methylated loci to the surrounding areas is called DNA neighborhood effect. This phenomenon which has been proposed to be involved in spreading abnormal hypermethylation is

supported by a study's findings revealing that DNA methylation can cause epigenetic silencing throughout a piece of chromosome of about 1Mb in size (Frigola et al. 2006).

The other force that is believed to drive abnormal methylation in CRC is the selective advantage. It is feasible to accept the concept of the selective advantage because different cancers are characterized by distinct methylation patterns. In addition to selective targeting and selective advantage, other models provide insight into aberrant DNA methylation adopted by cancer cells such as the dysregulated noncoding RNAs model. In humans, it is found that noncoding RNAs, microRNAs and piwi-RNAs can induce epigenetic alterations during cancer development by directly targeting DNMTs function (Garzon et al. 2009; Huang et al. 2010). However, the role of noncoding RNA in the human genome does not appear to be a generalized machinery for controlling DNA methylation. In contrast, abnormal DNA methylation seems to affect the expression of noncoding RNAs and cause them to be silenced, a mechanism that plays a significant role in CRC pathogenesis (Saito & Jones 2006).

In the context of epigenetic alteration, it has been found that abnormal DNA methylation may serve as an indicator for precancerous changes occurring in the abnormal tissue surrounding cancer. This concept is termed "field cancerization" and it is used to describe the development of surrounding areas of precancerous alterations. In the colon, the emergence of neoplastic cells after resection of the entire tumour indicates the effects of field cancerization which reflect the vulnerability of normal tissue to carcinogenesis (Jothy et al. 1996; Polley et al. 2006). However, to what extent the effect of the cancer field could spread is not known, even though some evidence suggests that its effect may involve the entire organ at which the primary tumour is established (Shen et al. 2007). In support of this concept, a single gene study revealed that abnormally methylated *MGMT* gene, a DNA repair gene, can be found in 50% of healthy tissue surrounding

primary tumours with the same abnormality. In contrast, a small percentage of about 7% of healthy tissues surrounding tumours carry a methylated *MGMT* promoter when the primary tumours do not contain methylated *MGMT* (Shen et al. 2005).

Besides single gene studies, the methylation status of a set of genes involving *SFRP1*, *SFRP2*, *APC*, *AXIN2* and *TUSC3* in histologically normal colonic mucosa has been evaluated. Interestingly the results showed that the methylation patterns of genes obtained from people with colorectal tumours significantly differ from those of people free of CRCs (Lao & Grady 2011). These methylation differences were used successfully to distinguish 79% of CRC patients and 88% of cancer-free patients (Belshaw et al. 2008). This has led to the identification of several panels of methylated genes in the normal colonic mucous membrane, wherein each time detectable concordance between gene methylation levels and patients' neoplasm state has been specified. In addition to the multiple genes studies, some evidence supporting the role of field cancerization as effector and indicator in CRC comes from assessing synchronous colorectal tumours (Ibrahim et al. 2010; Konishi et al. 2009). These types of tumours are shown to possess parallel DNA methylation patterns in numerous genes that are believed to be essential in the development of CRCs. Further evidence on field cancerization impact show the presence of a mutual relationship between methylation status of specific genes in the tumour-surrounding normal tissue and mutations in the tumour (Lao & Grady 2011). Such correlations can be obvious in the relationship between *K-ras* mutated tumours and hypermethylated *MGMT* gene in the adjacent normal tissue where it is believed that loss function of *MGMT* gene is a primary contributor of *K-ras* mutation (Hiraoka et al. 2010; Ramirez et al. 2008). This evidence supports that abnormal methylation can establish a predisposition for specific tumour associated mutations which in turn assist in disease propagation.

1.4.4.1 Role of abnormal methylation in CRC initiation and metastasis

Although hundreds of genes are found to be aberrantly methylated during the development of CRC, only a subclass appears essential for the pathogenesis of this disease (Kim et al. 2006; Rashid et al. 2001; Sunami et al. 2011). Figure 1 shows the common methylation genes involved in the adenoma-carcinoma sequence. Throughout the earliest steps in CRC formation which involves cancer initiation and progression, different sets of genes are methylated compared with those methylated during cancer metastasis. This variation indicates that gene methylation is a directed process, where each aberrantly methylated gene has specific role required for carcinogenesis. For instance, abnormal methylation of the DNA repair gene, *MGMT*, is found to be essential for promoting the progression of colonic adenoma to cancer through providing permissive environment for guanine-to-adenine transition mutations which are considered the most common mutations in *K-ras* (Kim et al. 2006; Rashid et al. 2001; Sunami et al. 2011). Aberrantly methylated genes are detectable at various stages of polyp to cancer sequence; normal mucosa, early adenomas, advanced adenomas, and adenocarcinomas, as well as in the mucosa of people with a high possibility of developing CRC. By comparing the methylation levels of genes during these stages, a substantial increase in gene methylation can be seen in early adenomas relative to normal mucosa, in advanced adenomas relative to early adenomas, and in adenocarcinomas relative to advanced adenomas (Esteller et al. 2000; Kim et al. 2006; Nosho et al. 2008; Sunami et al. 2011). Examples of methylated genes required for transformation of normal mucosa to aberrant crypt focus are *SLC5A8*, *MIMT1*, *MINT31, SFRP1*, *CDH13*, *CRBP1* and *RUNX3* (Kim et al. 2006; Rashid et al. 2001) while methylated genes required for transformation of abnormal crypt focus to polyp-adenoma are *P14*, *HLTF*, *ITGA4*, *CDH1* and *ESRI* (Lao & Grady 2011). Both sets of genes are believed to be essential for initiation and progression of CRC. In contrast, it is proposed that

genetic alteration is more likely to drive the process of CRC metastasis; however, some evidence suggests that methylation can play a role in this process. For example, abnormally methylated chemokine ligand, *CXLC12*, which frequently seen in CRC can induce the process of metastasis in colon cancer cell lines (Wendt et al. 2006). Other abnormally methylated genes found commonly in colon cancer and metastases compared to adenomas are TIMP3, CXCL12, ID4, and IRF8. It is believed that the abnormal methylation-induced inactivation of these genes confirms a clonal growth advantage acquired for metastasis. However, in general, abnormal gene methylation is involved more in the early steps of cancer development and less in later steps (Estecio et al. 2007; Kim, Lee & Sidransky 2010; Ogino et al. 2008).

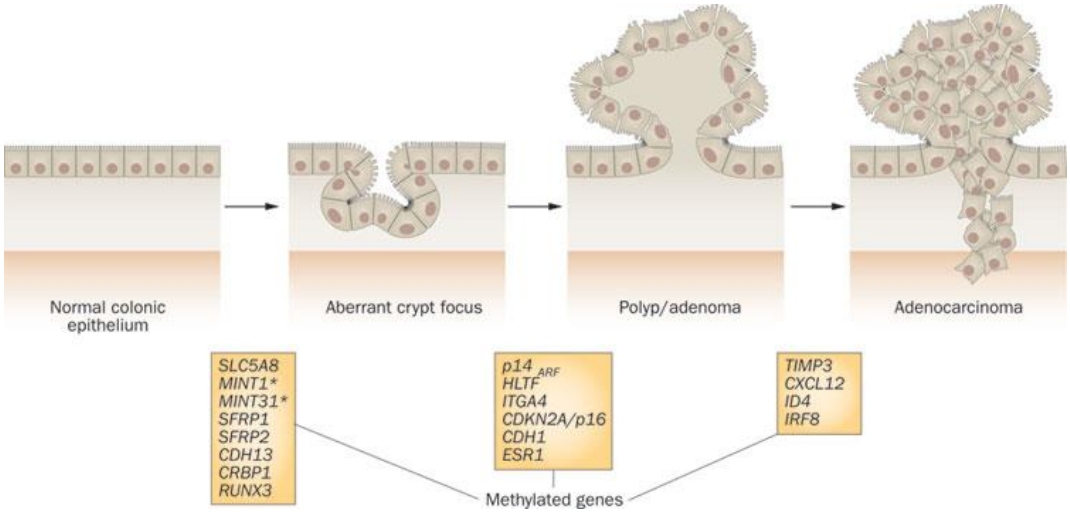

Figure 1. The commonly methylated genes during the adenoma-carcinoma sequence of CRC (Lao & Grady 2011).

1.4.5 Diagnosis of CRC

Several methods have been developed for CRC screening. These methods utilize the physiological and genetic abnormalities associated with CRC and/or adenoma presence. However, detection of CRC is still one of the great challenges facing researchers, physicians, and patients for different

reasons including; the early stage of CRC is often asymptomatic; lack of a gold standard diagnostic method and poor patient's adherence to CRC screening programs (Creeden et al. 2011; Summers et al. 2013). According to the US Preventive Services Task Force (USPSTF), the presence of an effective early screening method for CRC could decrease mortality (prevent 60% of CRC deaths) and boost the survival rate from 46 to 73% (National Cancer Institute 1999). Unfortunately, currently available screening methods are not accurate and sensitive enough to identify CRC at all stages; therefore, the majority of patients (75%) are found at middle or late stages, when they are diagnosed, and only 25% of cases could be diagnosed at early stages (American Cancer Society 2011).

1.4.5.1 Biomarkers as screening tools

Biomarkers represent biological molecules found in blood, other body fluids or tissues where their existence reflects a particular biological or pathological condition (Lange & Laird 2013). Cancer biomarkers could be either a molecule produced by cancer itself or created by the body as a response to the presence of cancer (Toth et al. 2012). Different sorts of biomarkers have been identified, related to nucleic acids, proteins, miRNAs and epigenetic alterations (Summers et al. 2013).

As mentioned previously, epigenetic changes, hypermethylation in particular, play important roles in the development of CRC. Analysis of cancer-specific DNA methylation patterns could facilitate the development of precise biomarkers which in turn can be used for detection, prognosis and diagnosis of CRC (Toth et al. 2012; Warren et al. 2011). The comparatively high frequency of abnormal methylation seen in CRC gives an advantage of utilizing it over other types of abnormalities as a biomarker for CRC detection. Another advantage of using abnormal methylation as a biomarker is that the organ-specific benign and inflammatory illnesses possess

unique methylation patterns that vary from that of malignant tumours of same organ (Laird 2003; Liggett et al. 2010; Liggett et al. 2011). Additionally, compared with gene expression profiles, DNA methylation profiles act as a stable source of molecular analytical and diagnostic information with patterns that can last longer without changes despite internal or external stimuli (Laird 2003).

Many methylated genes/loci have been evaluated as candidate biomarkers for diagnosis of CRC. For example, in a study that tested a panel of three hypermethylated genes (*TMEFF2*, *NGFR*, and *SEPT9*) in human plasma samples of healthy controls and CRC patients, *SEPT9* showed promising results as the most sensitive candidate (Lofton-Day et al. 2008). Since then, *SEPT9* gene has been validated by number of case/control studies as a biomarker for diagnosis of CRC where the sensitivity and the specificity of *SEPT9* assay in these studies ranged between 66-80% and 85-99%, respectively (Grutzmann et al. 2008; Toth et al. 2012; Warren et al. 2011). In addition to *SEPT9*, other hypermethylated biomarkers used for CRC detection include *SYNE1* and *FOXE1* genes. The combined sensitivities and specificities of *SYNE1* and *FOXE1* genes after being validated in two case-control studies were 56-58% and 90-91% respectively. However, the sensitivity of test for stage I CRC was 41% while for stage II CRC was 80% (Papadia et al. 2014) suggesting that use of the biomarkers for the earliest stage of CRC has low sensitivity.

Recently, characterization of a panel of 23 genes was performed by two novel methods of genome-wide methylation assessment (Mitchell et al. 2014a). The combined analysis identified candidate biomarkers including *BCAT1*, *COL4A2*, *DLX5*, *FGF5*, *FOXF1*, *FOXI2*, *GRASP*, *IKZF1*, *IRF4*, *SDC2* and *SOX21* that show elevated level and frequency of methylation in a high proportion of peripheral blood samples from CRC patients relative to healthy controls. Further validation of this panel of methylated DNA biomarkers was performed, and indicated that *BCAT1* and *IKZF1* are the most suitable candidates for use as CRC detection biomarkers (Mitchell et al. 2014a).

Despite the results of previous feasibility studies, the majority of available assays for screening CRC on the basis of evaluating DNA methylation biomarkers are able to detect late stage rather than early and non-invasive stages of the disease. Therefore, these assays may be more useful for measuring therapeutic response, prognosis and supporting clinical decisions (Pedersen et al. 2015a; Symonds et al. 2016).

1.4.5.1.1 The validation of *BCAT1* and *IKZF1* as methylated blood biomarkers.

To further validate the feasibility of using *BCAT1* and *IKZF1* as methylated DNA biomarkers, DNA samples were extracted from the blood of 144 healthy controls and 74 CRC cases and the methylation levels of these two markers were assessed by novel methylation-specific PCR assays. Hypermethylated *BCAT1* and *IKZF1* genes were identified in 48 (65%) and 50 (68%) of the 74 CRC cases respectively, while a very small proportion of healthy controls were positive for methylated forms of *BCAT1* and *IKZF1* (Pedersen et al. 2015b).

1.4.5.2 IKZF1

Ikaros family zinc finger protein 1, *IKZF1*, is the most studied member of Ikaros family of protein-encoding genes. This family expresses a number of important transcription factors including *IKZF3* (Aiolos), *IKZF2* (Helios), *IKZF4* (Eos), *IKZF5* (Pegasus) and *IKZF1*. Members of Ikaros family are characterized by the presence of zinc finger motifs, consisting of two β-strands and a α-helix that form a globular structure stabilized by a zinc atom surrounded by four amino acid (cysteine and histidine) residues (John & Ward 2011). Zinc finger motifs assemble to form two functional domains (Rebollo & Schmitt 2003). The first domain localizes at the N-terminus of protein and it is responsible for mediating the binding of protein to specific DNA sequences throughout the genome, while the second domain can be found at the C-terminus of protein and it

allows homological or heterogenetic dimerization between proteins of Ikaros family (Georgopoulos, Moore & Derfler 1992).

Full-length transcription of *IKZF1* gene generates an IK-1 isomer which contains four zinc finger motifs at N-terminus and two at C-terminus. However, alternative splicing events can form at least seven additional isomers, IK-2 through IK-8, characterized by different subsets of zinc finger motifs at N-terminus while sharing the same zinc finger domain at C-terminus as IK-1 (Georgopoulos 2002; Katia Georgopoulos, Susan Winandy & Avitahl 1997). Among the eight transcripts, IK-4 and IK-8 behave as dominant negative mutants because they have no zinc finger motifs at C-terminus which in turn disables their DNA binding ability (John & Ward 2011).

Early during embryonic development both hematopoietic and non-hematopoietic expressions of *IKZF1* gene were observed in lymphoid, myeloid and erythroid precursors (Dumortier et al. 2003; Kirstetter et al. 2002) and the developing striatal neurons (Agoston et al. 2007). In adults the expression of *IKZF1* gene was thought to be restricted to lymphopoietic tissues and peripheral blood leukocytes (Molnar et al. 1996). Initially, IKZF1 protein was recognized as a DNA-binding factor associated with specific DNA sequences at regulatory regions of T-cells (Georgopoulos, Moore & Derfler 1992). However, later IKZF1 proteins were found to play important roles in the regulation of T-lymphocytes, B-lymphocytes and natural killer cells production (Georgopoulos et al. 1994; Winandy, Wu & Georgopoulos 1995). At the molecular level, transcripts of Ikaros genes behave as either an activator or repressor of target gene expression. This double behavioral feature of IKZF1 proteins results from the multiple associations that IKZF1 proteins can make between each other as well as with several other proteins including ATP-dependent chromatin remodeler (Mi-2β), histone deacetylases (HDACs), metastasis-associated protein (MTA2) and RNA-binding proteins (Rbp). Assembly of these components with 10-12 molecules of IKZF1 proteins in

lymphocytes makes the nucleosome remodelling and deacetylation, NuRD, complex which has potential chromatin remodeling activity and can deacetylate histone (Georgopoulos 2002; Xue et al. 1998; Zhang et al. 1998). Moreover, some of Ikaros proteins can also be observed as part of SWI–SNF remodeling complex in association with Sin3 and CtBP which are coreceptors proteins (Koipally & Georgopoulos 2000; Koipally et al. 1999). As a repressor of gene expression, *IKZF1* participates in locating target gene to the pericentromeric-heterochromatin where inhibition of genetic expression takes place as a consequence of changing chromatin accessibility (Cobb et al. 2000). Another explanation is that the HDAC activity that is present in the NuRD complex tends to decrease chromatin accessibility by removing acyl groups, and subsequently prevents the expression of target genes such as inhibition of genes that promote myeloid differentiation early during hematopoietic progenitor (Nichogiannopoulou et al. 1999). On the other hand, downregulation of genes promoting lymphocytes differentiation in Ikaros-null stem cells and lymphocytes suggests that Ikaros-NuRD complex provides chromatin fluidity, a mechanism by which an equilibrium between accessible and non-accessible chromatin status is achieved (Kingston & Narlikar 1999). This mechanism could potentiate gene expression in distinct target sequences where transcriptional activators can be accommodated. Therefore, Ikaros can stimulate target gene expression via the action of its related chromatin remodeler through allowing an additional set of transcription factors such as histone acetyltransferases to overcome HDAC activity and provide an accessible chromatin state (Georgopoulos 2002; Nichogiannopoulou et al. 1999).

Few studies have discussed the role of *IKZF1* gene during cancer development. In one study that investigated the role of deregulated *IKZF1* gene in the development of stage IIIA non-small cell lung cancer, it was shown that the ectopic expression of *IKZF1* could inhibit the invasion and

metastasis of lung cancer, *in vitro*. In the same study, the downregulation of *IKZF1* expression was found to be correlated with cancer-induced hypermethylation status of the gene (Zhang et al. 2013). This result is consistent with Virely et al. (2010) findings which indicated that *IKZF1* gene can behave as a major tumour suppressor gene involved in human B-cell acute lymphoblastic leukemia. In another study aimed to validate DNA methylated markers for detection of pancreatic cancer, *IKZF1* gene was among the hypermethylated markers that highly discriminated pancreatic cancer cases from controls (Kisiel et al. 2015). In CRC, the effect of DNA methylation on Ikaros family (*IKZF1*, *IKZF3* and *IKZF2*) was investigated in a number of human CRC cell lines. Almost complete absence of hypermethylation within *IKZF3* and *IKZF2* promoters was observed while the promoter of *IKZF1* gene showed to have a very specific cancer-induced hypermethylation pattern. In part, it is believed that this happens because *IKZF1* gene promoter was found to be localized within a long-range epigenetic silencing region (LRES) which is a chromosomal region commonly seen in DNA of CRC cells in which CpG islands of several adjacent genes are concurrently hypermethylated and consequently transcriptionally repressed (Clark 2007; Frigola et al. 2006; Mayor et al. 2009). In the same study, ChIP analysis was used to recognize the possible direct gene targets of *IKZF1* products. The results highlighted some *IKZF1* target genes including *PTPN6, MEIS2, GPX7* and *NHLH1* which are found to overexpressed in CRC (Javierre et al. 2011). There is limited work on the methylation and gene expression of *IKZF1* in CRC tissue.

1.4.5.3 BCAT1

Branched-chain amino acids or (BCAAs) is a term used to describe the most hydrophobic amino acids, (leucine, isoleucine and valine), that contain aliphatic branched side-chains composed of a carbon atom attached to three or four hydrogen atoms (Harris et al. 2005). In eukaryotic cells, BCAAs are nutritionally essential, and they act as N2 donors in the peripheral tissues (Hutson,

Sweatt & LaNoue 2005). They participate in the formation and regulation of many proteins (Kimball & Jefferson 2004; Lynch et al. 2000). More specifically, they determine the structure of globular proteins and organize the interactions of transmembrane domains of membrane proteins with the lipid bilayers (Brosnan & Brosnan 2006). The BCAA leucine is a ketogenic amino acid that acts a precursor for ketones bodies through producing acetoacyl-CoA (Hutson, Cree & Harper 1978). BCAA metabolites, branched chain alpha-keto acids, can stimulate insulin secretion from β-cells of the pancreas (Zhou et al. 2010). In addition, in the brain BCAAs regulate the production of glutamate and γ-Aminobutyric acid that behave as neurotransmitter excitatory and neurotransmitter inhibitor respectively (Bak, Schousboe & Waagepetersen 2006; Bixel et al. 2001).

Unlike the other dietary amino acids that are metabolized in the liver, the majority of BCAAs escape the hepatic catabolism and enter the circulation system, so they reach their main catabolism sites including skeletal muscles, fatty tissue and brain (Fernstrom 2005; Hagenfeldt, Eriksson & Wahren 1980). The metabolism of BCAAs includes two initial enzymatic steps catalyzed by branched-chain amino acid transaminases (BCAT) and branched-chain keto acid dehydrogenases (BCKDs) respectively (Harris et al. 2005; Hutson, Sweatt & LaNoue 2005).

In humans, BCAT enzymes are homo-dimers composing of a small subunit (171 residues) and a large (183 residues) subunit that bind together through a loop connecting region (10 residues) (Conway et al. 2002). The active site of BCAT is located at the interface between the small and the large subunits (Yennawar et al. 2001). As with other catabolic enzymes, BCAT requires a co-factor termed pyridoxal-5'-phosphate (vitamin B-6) to be activated. Binding of vitamin B-6 allows conformational changes within the enzyme which in turn facilitates the binding of the substrate to the active site of the BCAT (Toney 2011).

Two isoforms of BCAT have been recognized in humans: mitochondrial BCAT (BCATm) and cytoplasmic BCAT (BCATc). Although BCAT isoforms have 58% homology, they differ in their location and enzymatic activity (Hutson 2001). The efficacy of BCATc isoform is higher than the BCATm isoform with a turnover rate 3-5 times faster. However, BCATc isoform is less common than BCATm isoform (Davoodi et al. 1998). Both isoforms are expressed during and after embryogenesis. The BCATc isoform exclusively can be found in the cytoplasm of the nervous system cells, in contrast, the BCATm is located in the mitochondria of most types of cells especially pancreatic cells (Hutson, Sweatt & LaNoue 2005).

BCAT1 gene expresses the cytoplasmic isomers of branched-chain amino acid transaminase (BCATc), and it is located at 12p12.1. Due to its critical function in BCAAs catabolism, BCATc is found to be associated with several cellular activities including cell growth, cell cycle progression, programmed cell death and differentiation (Rodriguez et al. 2003; Schuldiner et al. 1996).

In cancer, deregulation of *BCAT1* expression has been observed during the progression of various types of cancer indicating its roles in tumorigenesis. It has been found that upregulation of *BCAT1* expression is associated with growth and development of nasopharyngeal carcinoma cells where arrest of the cell cycle was observed in these cells following the knock down of *BCAT1* expression (Zhou et al. 2007). In the same study, it was reported that upregulation of *BCAT1* expression during the early stages of nasopharyngeal carcinoma occurred as consequences of gene amplification events within the 12p12 chromosomal region, where *BCAT1* is located, and transcriptional regulation through the overexpression of c-Myc transcription factor which can actively bind to *BCAT1* promoter (Zhou et al. 2007).

A relationship between *BCAT1* expression and the concentration of alpha-ketoglutarate substrate (α-KG) was shown in glioma tumours. In these tumours, a mutation in isocitrate dehydrogenase gene (IDH) is a frequent event. The mutated IDH uses α-KG as a precursor for producing 2-hydroxyglutarate and consequently downregulation of *BCAT1* through α-KG depletion (Tonjes et al. 2013). In the same study, it was also reported that a decrease in the catabolism of BCAAs through knocking down of *BCAT1* could lead to a reduction in the proliferation of a glioma cell line. This effect on glioma cell proliferation is thought to take place because blocking BCAA catabolism can cause reductions in the amount of branched chain Acyl-CoAs which are considered as precursors for mitochondrial ATP synthesis through to tricarboxylic acid cycle (Tonjes et al. 2013). The epigenetic regulation of *BCAT1* was also detected in epithelial ovarian cancer (Wang et al. 2015). It was observed that *BCAT1* overexpression occurs because of its promoter hypomethylation status, and blocking *BCAT1* expression can result in significant reduction in proliferation and migration of epithelial ovarian cancer (Wang et al. 2015). Silencing of *BCAT1* is believed to be DNA methylation-dependent, happening as either a late event during ovarian cancer development (Wang et al. 2015) or an early event during nasopharyngeal carcinoma pathogenesis (Zhou et al. 2013). While the *BCAT1* promoter region has been found to be hypermethylated in CRC tissue (Mitchell et al. 2014a), no work has looked at how this may affect gene expression.

1.4.6 Radiotherapy for cancer treatment

Many types of treatments exist for individuals with CRC including surgery, cryosurgery, chemotherapy, radiotherapy and targeted therapy. However, the majority of the rectal cancer cases are treated by combining surgery with chemo/radiotherapy (Baskar et al. 2012). Recently, the use of radiotherapy has expanded with an enormous development in treatment planning, where best fractionation and accuracy of dosage to the target volume are essential factors for reducing the

side-effects and boosting the likelihood of cancer management (ICRU 2010). Radiotherapy is a very cost effective treatment where it accounts for only 5% of the entire cost of cancer care annually (Ringborg et al. 2003), and it contributes to about 40% towards curative treatment (Barnett et al. 2009). Moreover, it has been estimated that about 50% of cancer patients are subjected to radiotherapy at a certain point during their course of the disease (Begg, Stewart & Vens 2011; Delaney et al. 2005) making radiotherapy one of the most frequently used treatment modalities for cancer clinical control. In some cancer cases, radiotherapy is used prior to surgery. The aim of this treatment "neoadjuvant therapy" is to shrink the tumour when its size and/or location makes it hard to be removed completely. Also, radiotherapy can be used as "adjuvant therapy" after surgery to minimize the risk of the tumour recurrence (destroy any cancerous cells that may have been left behind after surgery) especially rectal cancers. Additionally, radiotherapy can be applied as "palliative radiotherapy" to slow down cancer cells growth and development in patients who are not healthy enough for surgery, and to relieve symptoms of advanced cancer such as bleeding, pelvis or rectum pain and intestinal blockage (American Cancer Society 2014).

Radiotherapy is an application of high-energy ionizing radiation (IR) such as x-rays and protons to destroy cancer cells. In principle, IR is a physical agent that forms electrically charged particles (ions) through the removal of electrons from atoms and molecules. When these ions pass through cells and tissues, they deposit high levels of energy blocking cellular growth and development (Jackson & Bartek 2009). It is well known that IR causes DNA single and double strand breaks (Baskar et al. 2012). Accumulation of IR-induced DNA breaks leads to cell cycle arrest and chromosomal rearrangement in both cancerous and non-cancerous cells (Hall et al. 2006). Even though the IR-induced DNA damages are not limited to the cancer cells, radiotherapy, as mentioned before, is one of commonly used tools for cancer management (Erven & Van

Limbergen 2007; Little 1999, 2000). The rationale of harnessing the IR as a therapeutic tool is that the rapidly dividing cancer cells lose their capacity to perform effective DNA repair, and also they generate more DNA breaks, leading to make them more vulnerable to radiotherapy (Bernier, Hall & Giaccia 2004). On the other hand, the relatively slowly proliferating normal cell has effective DNA repair mechanisms representing vital lines of defense against damage caused by exposure to radiotherapy, and consequently normal cells become less radiosensitive (Baskar et al. 2012; Desouky, Ding & Zhou 2015; Kim et al. 2013). Therefore, applying radiation for cancer treatment stimulates multiple responses in the cells where several proteins involved in cell death and DNA damage pathways are produced to increase the radiosensitivity of cancer cells and induce their death, and decrease the radiosensitivity of normal cells and promote their survival.

1.4.6.1 Bystander effect of radiotherapy

Improvements in the efficacy of radiotherapy to killing cancer cells rather normal cells have been achieved, allowing the application of radiotherapy effectively for managing tumours throughout the body especially localized tumours (Baskar 2014). However, a growing body of evidence has shown that radiotherapy can cause damage to the tumour's adjacent tissue as well as for tissue located far from the radiation track, a phenomenon termed as bystander effect (Nagasawa & Little 1992; Shao et al. 2004; Suzuki & Yamashita 2014). Bystander effect has been identified in several *in vitro* and *in vivo* studies where IR-induced chromosomal aberrations can be seen not only in the irradiated cells but also in the non-irradiated cells (Bentzen 2006; Seymour & Mothersill 1997). One way of transmission of radiation-induced damage signals is thought to be through cell gap junction-mediated intercellular communication occurring between adjacent cells (Azzam et al. 1998). It is also believed that the exposure of tissue to IR can promote the release of a range of soluble signaling molecules including cytokines to the non-irradiated cells. These signaling

molecules can initiate various cellular and microenvironmental signaling cascades which in turn trigger several damaging responses in the distant non-targeted cells (Hei et al. 2011; Ivanov et al. 2010). Recently several candidate mediators for the bystander effect have been observed including TGF-β1 (Iyer & Lehnert 2000), TNF-α and IL-8 (Prise & O'Sullivan 2009) which can stimulate cell growth, DNA damage, and cell death respectively. The effects of radiation on non-irradiated cells are known as delayed genomic instabilities that are observed many generations after exposure of targeted cells to radiation resulting in generating de novo in the progeny of non-irradiated cells (Gow et al. 2010; Ponnaiya, Cornforth & Ullrich 1997; Suzuki et al. 2003).

1.4.6.2 Radiation and epigenetic modifications

As mentioned previously DNA methylation is responsible for activation of tumour oncogenes and suppression of tumour repressor genes that eventually lead to cancer development. Changes in DNA methylation patterns in response to radiation exposure has been discussed in several studies. It is important to determine the influence of radiation on DNA methylation patterns as it has a potential impact on responses to current and future treatments through increasing or decreasing radio-resistance of targeted tumour cells (Zielske 2015). By assessing global DNA methylation in mouse models following radiation exposure, a global loss of DNA methylation associated with a decrease in the expression level of methyltransferases (DNMT1 and DNMT3a) and methyl CpG binding proteins were observed (Loree et al. 2006; Pogribny et al. 2004; Raiche et al. 2004). These results pave the way for further analysis of the potential genomic instabilities induced by exposure of cancer cells to radiotherapy. Further studies, using Chip technology incorporated with pathway analysis to determine radiotherapy-induced DNA methylation changes at specific loci reported that some genes involved in cell cycle control, apoptosis and DNA repair pathways were differentially methylated in dosage and time-dependent manners (Bibikova et al. 2011; Zielske

2015). Hypomethylation and hypermethylation of genes involved in cell cycle control were observed following cell exposure to low doses (2Gy) and high doses (6Gy) of radiation respectively. This result correlates with the recovery of the G2/M checkpoint following a low radiation dose, and with loss of the G2/M checkpoint following a high radiation dose (Zielske 2015). Similarly, in *in vitro* studies, global DNA methylation changes and loci specific differential methylation were observed following the exposure of breast cancer cell lines to radiation with and without a recovery period. Interestingly, a degree of recovery in methylation patterns associated with no changes in radio-sensitivity of irradiated cells was seen after the recovery period (Antwih et al. 2013; Kuhmann et al. 2011). The level of DNA methylation before and after radiotherapy also has been analyzed in biopsies of breast cancer by using Illumina Infinium 27 k microarray. Between the irradiated and non-irradiated groups, 82 genes were found to be differentially methylated where the major variation in DNA methylation was observed in inflammatory genes (Halvorsen et al. 2014). Some of these genes were chosen for further study since changes in their methylation patterns following radiotherapy were expected to be associated with unique clinical responses. These changes could be used as indicators to predict patients responses to radiotherapy as well as guide physicians to determine dosage required to achieve the desired results (Smits et al. 2014; Zielske 2015).

1.5 Knowledge gaps, aims and hypotheses

Previous studies have shown that cancer-induced methylation can deregulate levels of gene expression. Overall hypomethylation events associated with genes overexpression can be seen in the genome of cancer cells while hypermethylation events (silencing) can be observed in genes involved in cell cycle regulation, DNA repair and cancer invasion inhibition (Phillips 2008). Similarly, it has been found that development of CRC causes hypermethylation of *BCAT1* and

IKZF1 genes (Mitchell et al. 2014b) where increasing levels of circulating methylated *IKZF1* and *BCAT1* were observed as CRC stage progresses (Pedersen et al. 2015c). However, it is not known how this hypermethylation affects the expression of *BCAT1* and *IKZF1* genes.

Moreover, it is well known that exposure of cancer cells to cancer treatment (radiotherapy) can lead to global DNA hypomethylation associated with a decrease in the expression level of methyltransferases (Loree et al., 2006; Pogribny, Raiche, Slovack, & Kovalchuk, 2004; Raiche, Rodriguez-Juarez, Pogribny, & Kovalchuk, 2004). However, is unknown how cancer treatment affects methylation levels and gene expression of *BCAT1* and *IKZF1*. Therefore, we hypothesize that expression of *BCAT1* and *IKZF1* will be decreased in hypermethylated CRC tissue. Also, we hypothesize that cancer treatment will reduce the methylation level of these two biomarkers (*BCAT1* and *IKZF1* genes) within CRC tissue and be associated with an increase in the levels of expression of *BCAT1* and *IKZF1* genes.

The specific objectives of the project were:

1. Compare the methylation levels of *BCAT1* and *IKZF1* in CRC tumour samples with patient's pathological data.

2. Detect the effects of methylation on the gene expression levels of *BCAT1* and *IKZF1* in colorectal tumour samples and the para-tumour tissue samples.

3. Determine the effects of cancer treatment on the methylation level of *BCAT1* and *IKZF1* using colorectal tumour samples and para-tumour tissue samples.

4. Determine the effects of cancer treatment on the gene expression levels of *BCAT1* and *IKZF1* in colorectal tumour samples and the para-tumour tissue samples.

1.6 The clinical significance of the study

The presence of hypermethylated *BCAT1* and *IKZF1* in the circulation has been recently shown to indicate the presence of CRC (Pedersen et al. 2015b). It has also been demonstrated that blood samples will become negative for these methylated biomarkers following tumour resection (Pedersen et al. 2015b). However, it is not known whether the blood test could also be used to assess and monitor response to cancer treatment. Therefore, by determining how the levels of hypermethylated *BCAT1* and *IKZF1* in colorectal tumours may change following neoadjuvant treatment, we will be able to develop the blood test to assess response to treatment and help physicians to make important clinical decisions.

In addition, the function of *BCAT1* and *IKZF1* in CRC has not yet been extensively studied. Studying the expression levels of *BCAT1* and *IKZF1* in colorectal tumour tissue and comparing it with those levels in para-tumour tissue samples has two advantages. First, it is the key to understanding the roles that *BCAT1* and *IKZF1* play in colorectal tumour growth and development. Second, it increases the feasibility of making *BCAT1* and *IKZF1* as future candidates for targeted cancer therapies and prognostic markers to monitor treatment response.

Chapter 2. Materials and methods

2.1 Patient population and tissue samples

Tumour and para-tumour tissue samples of 50 patients aged between 39 and 85 years (median age 65 years), who had been diagnosed with invasive CRC (92% of patients presented with local disease; stage I, II or III at diagnosis, while the remainder had metastases), were randomly selected for analysis in this study. Patients (44% female and 56% male) underwent tumour surgical resection at either Repatriation General Hospital (Daw Park, SA) or Flinders Medical Centre (Bedford Park, SA). Samples were collected from the resected tissue from a viable part on the luminal surface of the tumour, and from non-tumour tissue at least 5mm proximal or distal to the tumour (range 5-850mm, Table 3). The surgical specimens were stored in RNAlater (Qiagen) for at least 4 hours before they were transferred into new vials and stored at -80 °C within the joint Flinders Medical Centre and Repatriation General Hospital tissue bank. Samples were collected over a period of six years (between 2010 and 2016). Clinicopathological features of patients enrolled in this study, and the distance that the normal tissue was sampled from the tumour are presented in Table 3. Some patients of the 50 (n = 14) were subjected to different regimes of radiotherapy and chemotherapy prior to surgical resection. The average duration of neoadjuvant therapy was 34.7 days (range between 7-42 days), and the average time prior to surgical resection was 68.7 days (range between 13-96 days; Table 3). The study had ethics approval from the Southern Adelaide Clinical Human Research Ethics Committee (approval number 134.045). The trial is registered at Australian and New Zealand Clinical Trials Registry, trial registration number 12611000318987. All patients gave written informed consent prior to any samples being collected.

Table 3. Clinicopathological features of 50 patients with colorectal cancer.

Patients details			Tumour stage				Tumour features				Collection of samples		Neoadjuvant therapy		
ID	Gender	Age	T	N	M	TNM	Site	Size (mm)	No. of lymph nodes affected	Differentiation	Distance between tumour and para-tumour tissues (mm)	Location of para-tumour tissue in relation to tumour	Type	Duration (day)	Time until surgery (day)
3418	Male	70	3	0	0	IIA	Rectum	80	0/15	Moderate	295	proximal	25 x 2.0Gy	32	103
3474	Female	70	3	1b	1	IVA	Rectum	20	2/17	Moderate	300	proximal	25 x2.0Gy + Capecitabine	35	88
1811	Male	52	2	0	0	I	Rectum	36	0/17	Moderate	350	proximal	28 x 1.80Gy	37	98
3024	Female	50	3	1	0	IIIB	Rectum	20	1/14	Moderate	55	distal	28 x 1.80Gy	41	743
28	Male	70	1	0	1	IVA	Rectum	1.5	0/20	Moderate	45	Distal	28 x 1.80Gy + 5FU	37	76
560	Male	60	3	1	0	IIIB	Rectum	18	2/15	Moderate	40	Distal	28 x 1.80Gy + 5FU	38	62
708	Male	79	2	0	0	I	Rectum	60	0/24	Moderate	400	proximal	28 x 1.80Gy + 5FU	43	57
822	Male	71	1	0	0	I	Rectum	30	0/8	not stated	20	Distal	28 x 1.80Gy + 5FU	38	62
926	Male	79	2	1c	0	IIIA	Rectum	30	0/9	Moderate	30	Distal	28 x 1.80Gy + 5FU	37	99
965	Male	55	3	1a	0	IIIB	Rectum	40	1/10	Moderate	55	distal	28 x 1.80Gy + 5FU	37	63
2298	Male	49	3	1b	0	IIIB	Rectum	40	2/18	Moderate	515	proximal	28 x 1.80Gy + 5FU	37	61
3153	Male	76	Tis	0	0	0	Rectum	35	0/17	Poor	350	proximal	28 x 1.80Gy + 5FU	37	88
1411	Male	52	4b	0	1	IVA	Rectum	25	0/6	Moderate	160	proximal	28x1.80Gy (unknown chemo)	38	62
1010	Female	49	3	0	0	IIA	Rectum	25	0/7	Moderate	45	distal	5 x 5Gy	6	58
27	Female	61	3	1	0	IIIB	Hepatic flexure	40	2/28	Moderate	140	proximal	None		
235	Male	46	2	0	0	I	Rectum	35	0/11	Moderate	5	distal	None		
396	Male	74	4b	1c	0	IIIC	Rectum	50	0/8	Poor	50	Distal	None		

495	Male	85	3	0	0	IIA	Rectum	30	0/14	Moderate	100	proximal	None
501	Female	65	3	0	0	IIA	Sigmoid	35	0/17	Moderate	30	proximal	None
644	Female	73	3	0	0	IIA	Ascending colon	100	0/28	Moderate	85	proximal	None
689	Female	75	1	0	0	I	Rectum	11	0/1	Moderate	25	Not-documented	None
757	Female	65	2	1	0	IIIA	Ascending colon	50	1/23	Moderate	50	proximal	None
780	Female	81	1	1	0	IIIA	Ascending colon	10	2/12	Moderate	70	proximal	None
804	Female	52	3	2b	0	IIIC	Sigmoid	35	12/29	Moderate	55	Distal	None
861	Female	79	2	1	0	IIIA	Rectum	55	1/20	Poor	5	proximal	None
918	Female	52	3	0	0	IIA	Transverse colon	45	0/15	Moderate	110	Distal	None
924	Female	63	2	0	0	I	Sigmoid	20	0/18	Moderate	250	proximal	None
966	Male	85	4b	0	0	IIC	Rectum	50	0/7	Moderate	60	distal	None
982	Male	64	4a	1a	1	IVB	Splenic flexure	40	1/9	Moderate	130	proximal	None
1056	Male	69	1	0	0	I	Caecum	35	0/17	Poor	20	distal	None
1279	Male	67	3	0	0	IIA	Sigmoid	40	0/16	Moderate	35	distal	None
1340	Female	46	4a	1	1	IVA	Rectum	35	1/14	Moderate	225	proximal	None
1515	Female	81	3	0	0	IIIA	Transverse colon	50	0/17	Moderate	80	distal	None
1623	Male	72	3	0	0	IIA	Hepatic flexure	65	0/16	Moderate	70	distal	None
1681	Male	55	3	0	0	IIA	Rectum	70	0/23	Moderate	45	distal	None
1784	Female	63	3	1	0	IIIB	Ascending colon	40	2/12	Moderate	130	proximal	None
1979	Female	47	4a	2	0	IIIC	Rectum	30	4/17	Moderate	5	proximal	None
2322	Male	59	3	1	0	IIIB	Transverse colon	30	0/18	Moderate	5	proximal	None

3002	Female	44	2	0	0	I	Caecum	100	0/23	Moderate	240	distal	None
3017	Male	54	4a	1	1	IVA	Sigmoid	60	3/18	Moderate	70	proximal	None
3057	Male	46	3	2	1	IVA	Sigmoid	70	8/33	Moderate	850	proximal	None
3079	Male	61	2	0	0	I	Ascending colon	40	0/12	Poor	45	distal	None
3108	Female	50	4a	2a	0	IIIC	Descending colon	65	5/25	Moderate	30	distal	None
3185	Female	78	3	0	0	IIA	Rectum	38	0/15	Moderate	60	proximal	None
3317	Male	85	2	0	0	I	Caecum	25	0/12	Moderate	Not-documented	Not-documented	None
3393	Male	63	3	0	0	IIA	Sigmoid	70	0/38	Moderate	25	distal	None
3414	Female	65	1	0	0	I	Transverse colon	25	0/10	Moderate	55	distal	None
3424	Female	39	4a	1	1	IVA	Descending colon	40	1/13	Moderate	140	proximal	None
3446	Male	70	3	0	0	IIA	Rectum	60	0/29	Moderate	70	proximal	None
16	Male	65	4	0	0	IIB	Sigmoid	100	0/60	Poor	5	distal	None

5FU represents Fluorouracil (a chemotherapy drug).

2.2 DNA Methylation analysis

2.2.1 DNA extraction

DNA was extracted from colorectal tumour samples (n = 50) and the adjacent para-tumour samples (n = 50). Extraction of genomic DNA was conducted using a DNAeasy® Blood & Tissue kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer instructions. Briefly, tissue samples of approximately 15 mg were used for DNA extraction after being cut by a scalpel into small pieces. Samples were then lysed by mixing with 180 µL ATL buffer and 40 µL proteinase K (Qiagen) and incubated in a heat block for 4 hours at 56°C, vortexing every 30 minutes. DNA was prepared for column separation by adding 200 µL of AL buffer (Qiagen); containing chaotropic salts (guanidinium hydrochloride) and 200 µL of 100% ethanol to the lysate. The mixture was thoroughly vortexed and then transferred into a DNeasy Mini spin column, (Qiagen), containing silica membrane to which DNA can be adsorbed. The separation assembly, which consisted of spin column and collection tube was centrifuged at 6,000 x g for 1 minute. The flow-through was discarded, and the DNeasy Mini spin column was placed in a new collection tube. Samples were washed by first adding 500 µL of AW1 buffer (Qiagen) to the spin column and centrifuging, then 500 µL of AW2 buffer (Qiagen) to remove any remaining impurities. Centrifugation speeds were 8,000 x g for 1 min and 18,000 x g for 4 minutes respectively. After each washing step, the flow-through was discarded and the washed column was placed in a new labeled 1.5 mL microcentrifuge tube. AE buffer (100 µL, Qiagen) was directly added to the membrane of the DNeasy Mini spin column and DNA was released and collected by centrifugation at 6000 x g for 1 minute.

2.2.2 DNA quantification

DNA concentrations were quantified with the NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) by measuring light absorbance at wavelength 260 nm. Additionally, the quality of DNA extraction and the presence of contaminants such as ethanol and guanidinium hydrochloride were assessed by measuring 260/280 nm and 260/230 nm ratios. An amount of 2 µl of each sample was used for quantification after blanking the NanoDrop using AE buffer (Qiagen). Extracted DNA was stored at -80°C until required for further processing.

2.2.3 Bisulfite conversion treatment

To allow for analysis of methylation status of CpG dinucleotides of *BCAT1* and *IKZF1*, all extracted DNA was first bisulfite converted using EpiTect® Fast Bisulfite Conversion kit (Catalog number 59824, Qiagen GmbH, Hilden, Germany) according to manufacturer instructions. Briefly, 500 ng of DNA was diluted in 20 µL of nuclease free water (Qiagen), mixed with 85µL of Bisulfite salt solution (Qiagen), and 35µL of DNA Protect Buffer (Qiagen) in a 200 µL tube, and placed in an MJ Mini thermocycler (BioRAD) for 30 minutes. The DNA was heated in a thermocycler as follows; 95°C for 5 minutes, 60°C for 10 minutes, 95°C for 5 minutes and 60°C for 10 minutes. The bisulfite acts by converting unmethylated cytosines into uracil, leaving the methylated cytosines unchanged and the DNA Protect Buffer minimises DNA degradation due to harsh conversion conditions, high temperature and alkaline pH. Once the heating cycle was completed, the bisulfite reaction was transferred to a new 1.5 mL tube after being centrifuged briefly. A volume of 310 µL of Buffer BL, containing guanidinium hydrochloride (Qiagen), followed by 250 µL of 100% ethanol, was added, and the mixture was centrifuged and pulse vortexed for 15 seconds. The mixture was transferred into a MinElute DNA spin column comprising a silica pad (Qiagen) and washed with 500µL BW buffer (Qiagen) and then with 250 µL ethanol to remove

any impurities. Each time the spin column was centrifuged at 18,000 x g for 1 minute and reinserted in a collection tube after discarding the flow-through. The final step of cytosine conversion was achieved by incubating the spin column with 500µL of Buffer BD (Qiagen) for 15 minutes at room temperature. The spin column was washed twice with 500µL BW buffer (Qiagen) and once with 250µL ethanol and centrifuged at 18,000 x g for 1 minute between each addition to remove any contaminants. The DNA was eluted into a 1.5 mL tube by adding 40 µL Buffer EB (Qiagen) directly onto the centre of the spin column membrane. The concentration of DNA conversion was measured by NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) as previously described.

2.2.4 Real time-PCR for methylation status

The methylation status of CpG dinucleotides of *BCAT1* and *IKZF1* was assessed by using a QuantiTect Multiplex PCR NoROX Kit (Catalog number 204743, Qiagen GmbH, Hilden, Germany) according to manufacturer instructions, but with some modifications. PCR reactions were performed using a Rotor-Gene® Q instrument (Qiagen). Each PCR reaction included a standard curve, no template controls and the bisulfite converted DNA of either the tumour or para-tumour samples. The standard curve was based on 6-fold serial dilution of 5ng of bisulfite converted CpG methylated genomic DNA (gDNA) of a Jurkat cell line (Acute T-cell leukemia; NEW ENGLAND BioLabs, Arundel, Qld, Australia) with nuclease free water containing 2 ng/µL of RNA carrier (Qiagen) as described in Table 4. The no template control wells were run in duplicate and they included only nuclease free water containing 2 ng/µL of RNA carrier. The bisulfite converted DNA samples were diluted by nuclease free water to obtain a final concentration of 1 ng/µL. Primer sets and probes used for amplifying specific methylated CpG dinucleotides within the promoter sequences of *BCAT1* and *IKZF1,* and specific non-methylated

CpG dinucleotides within the promoter sequences of β-actin (*ACTB*) were previously designed by Clinical Genomics Pty Ltd, (North Ryde, NSW, Australia). Primers were designed to target methylated CpG sites in regions spanning 102- or 95- nucleotides, either within, or just upstream of the first exon of the *BCAT1* and *IKZF1* genes, respectively (Pedersen et al. 2015b) (Figure 2), and ordered from Geneworks Pty Ltd (Thebarton, SA, Australia; Table 5). The working concentration (6 nmol) of primer pairs and probes was prepared by adding nuclease free water.

The real-time PCR amplification assays were performed in duplicate in a final PCR volume of 15 µL. The amplification mixtures contained 10 µL of QuantiTect Multiplex PCR NoROX Master Mix (Qiagen), 1.25 µL of methylated primers and probes of either *BCAT1*, *IKZF1* or *ACTB* gene and 5 µL of bisulfite converted DNA sample; nuclease free water was used to obtain the final reaction volume (15 µL), if required. The methylation status of *BCAT1* and *IKZF1* genes were assayed in a duplex PCR assay while methylated *ACTB* gene was assayed in a separate PCR tube. The cycling conditions comprised of 15 min 95°C for activation of polymerase followed by 50 cycles comprising of 60 seconds at 94°C for denaturation and 60 seconds at 60°C for combined annealing/extension.

Table 4. Serial dilutions of bisulfite-converted Jurkat gDNA standard curve used in the methylation PCR assay.

	Standard 1	**Standard 2**	**Standard 3**	**Standard 4**	**Standard 5**	**Standard 6**	**Standard 7**
Concentration of bisulfite-concerted Jurkat gDNA (pg/µL)	1000	400	160	64	25.6	10.24	4.1

nuclease free water containing 2 ng/µL of RNA carrier was used for dilution.

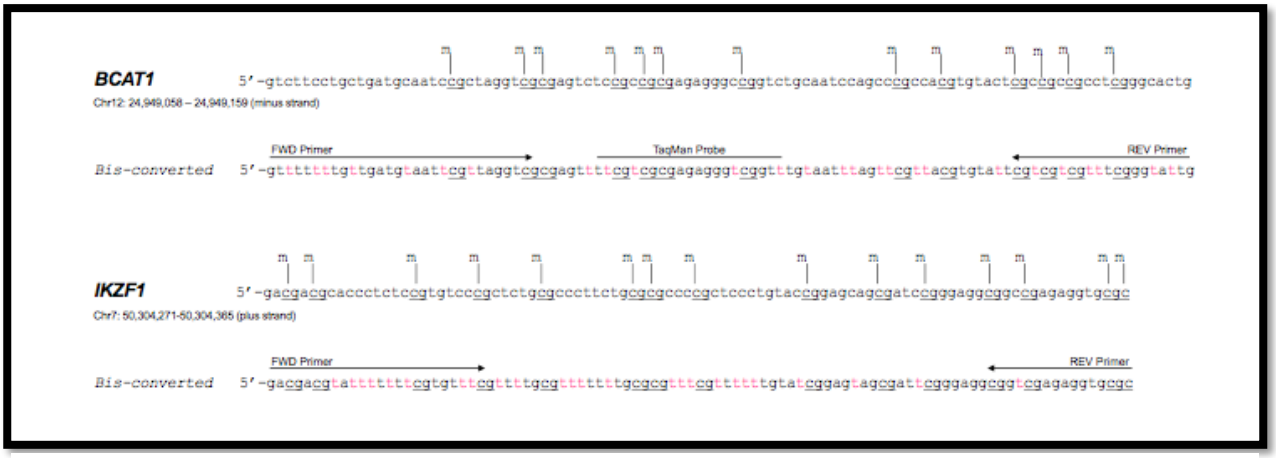

Figure 2. DNA sequences (upper sequence) and resulting bisulfite-converted fully methylated sequences (lower sequence) of *BCAT1* and *IKZF1* used for methylation PCR assay. Underlined: methylated cytosines (marked with a "m") residing in CpG sites. Red: unmethylated single cytosine residues converted to thymidines subsequent to bisulfite conversion and PCR. Arrows: bisulfite conversion and methylation specific forward and reverse primers (Pedersen et al. 2015b).

Table 5. List of nucleotide sequences (primers and probes) used in the methylation PCR assay.

Target genes		Methylate primer sequence (5' – 3')	Probe sequence (5' – 3')	Fluorophores and Quenchers
BCAT1	F	GTTTTTTTGTTGATGTAATTCG TTAGGTC	TTCGTCGCGAGAGGGTCGG TT	5: HEX 3: BHQ-1
	R	CAATACCCGAAACGACGACG		
IKZF1	F	GACGACGTATTTTTTTCGTGTT TC	TTTGTATYGGAGTAGYGAT TYGGGAGG	5: FAM 3: BHQ-1
	R	GCGCACCTCTCGACCG		
ACTB	F	GGAGTTTTTGTTTTTTGGTTAG TTG	ATGGAGGTTTAGTGGTAAT ATAGGTTTTGTTTGG	5: FAM 3: BHQ-1
	R	CAAAATAAAATACAAAACA AACCTAATCC		

Y stands for pyrimidine (a 50/50 mix of T and C).
F indicates forward sequence and R indicates reverse sequence.

2.2.5 Quantification of methylated DNA

The data was acquired during the combined annealing/extension step of each cycle on green and yellow channels of the Rotor-Gene® Q instrument. The probes of *IKZF1* and *ACTB* genes emitted florescence signal detectable on the green channel while *BCAT1* gene florescence signal was detected on the yellow channel. Analysis of the methylation PCR results was performed using Rotor-Gene Q Series Software (version 2.0.2). Ct values were established by setting a threshold just above the background fluorescence of the no template control for each primer/probe set. The absolute amount of methylated *BCAT1* and *IKZF1* in each sample (colorectal tumour and para-tumour) was calculated from the linear regression line fitted to the Ct values of the methylated Jurkat gDNA standard curve. The concentration of non-methylated *ACTB* was also determined in the same way. The amount of methylated DNA of *BCAT1* and *IKZF1* was normalized against the absolute amount of non-methylated DNA of *ACTB* gene (reference gene) in the corresponding sample and expressed as a percentage (%). Samples were considered to have no methylation if there was no amplification (no increase in fluorescence intensity) of either *BCAT1* and *IKZF1* within 45 cycles but there was amplification for *ACTB* as previously established for blood samples (Pedersen et al. 2015a).

2.3 Gene expression analysis

2.3.1 RNA extraction

Total RNA was extracted from tumour and para-tumour tissue samples by using a TRIzol® Plus RNA Purification Kit (Catalog number 12183555, Life Technologies, Mulgrave, Australia) under conditions indicated by the manufacturer. RNase away spray (Life Technologies) was used to decontaminate reagents, pipettes and tubes, and the extraction was performed in an RNase free fume hood. All steps of the extraction process were performed on ice. Tissue samples of size range

between 20 to 30 mg were cut into fine pieces using scalpels. Lysates were then prepared by homogenizing the samples in 1 mL of Trizol® reagent using a tissue homogenizer (G50 Tissue Grinder; Coyote Bioscience, Beijing, China) and left for 5 minutes at room temperature to allow complete dissociation of nucleoprotein complexes. The trizol contained a mixture of guanidinium thiocyanate, phenol and other components to facilitate RNA isolation and protection. Phase separation was performed by adding 200 µL of chloroform (Thermo Fisher Scientific) to each sample which was then incubated for 3 minutes at room temperature after shaking vigorously by hand for 15 seconds. Samples were then centrifuged at 12,000 × g for 15 minutes at 4°C. This process separated the mixture into lower red phenol-chloroform phase, an interphase and upper colorless aqueous phase. After centrifugation, a volume of 400 µL of the upper clear layer, containing RNA, was collected, placed in a new 1.5 mL vial and mixed with 400 µL of 70% ethanol to obtain a final ethanol concentration of 35%. Further purification of total RNA was performed by transferring 700 µL of ethanol-containing-sample to a Spin Cartridge (Life Technologies) containing silica membrane and assembled in a collection tube. The Spin Cartridge was then centrifuged at 12,000 × g for 15 seconds at room temperature and the flow–through was discarded. A 700 µL volume of washing buffer I (Life Technologies) was added followed by centrifugation at 12,000 × g for 15 seconds at room temperature and discarding the flow-through. After being inserted into a new collection tube, the spin cartridge was washed twice with washing buffer II (Life Technologies); each time a volume of 500 µL of washing buffer II was added followed by centrifugation at 12,000 × g for 15 seconds and the flow-through was discarded. An extra centrifugation step was performed for 1 minute at 12,000 x g to allow complete removal of washing buffer II. RNA was then eluted into a new labeled 1.5 mL centrifuge tube by adding 100

µL of RNase-free water (Life Technologies) followed by centrifugation at 12,000 × g for 2 minutes.

2.3.2 RNA quantification

The concentration of RNA was quantified with a NanoDrop 8000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) by measuring light absorbance at wavelength 260 nm as previously described. Additionally, the quality of RNA extraction and the present of contaminants such as ethanol and guanidinium thiocyanate were assessed by measuring 260/280 nm and 260/230 nm ratios. An amount of 2 µL of each sample was used for quantification after blanking the NanoDrop using RNase-free water (Invitrogen kit). Depending on the concentration of RNA, samples were stored at -80 °C in aliquots of 10 or 5 µL until required for further processing.

2.3.3 Preparation of cDNA

Gene expression of *BCAT1* and *IKZF1* was assessed through measuring mRNA levels with reverse transcription PCR (RT-PCR). Total RNA for all samples was subjected to reverse transcription using a QuantiNova™ Reverse Transcription Kit (Catalog number 205411, Qiagen GmbH, Hilden, Germany) in which Oligo-dt and random hexamer primers were used to achieve successful reverse transcription at 3' and 5' ends of target sequences. Before being reverse transcribed, 1 µg of RNA diluted in nuclease free water was incubated with 2 µL of Genomic DNA Removal Mix (Qiagen) in a final volume of 15 µL at 45°C for 2 minutes to reduce contaminating gDNA. The gDNA-free total RNA was mixed with reverse-transcription master mix containing 1 µL of Reverse Transcription Enzyme (Qiagen) and 4 µL of Reverse Transcription Mix (Qiagen) in a final volume of 20 µL and placed in the thermocycler (BioRAD). The temperature was first set to 25°C for 3 minutes to allow primers to anneal to their complementary sequences, followed by 10 minutes at 45°C to start the synthesis of a single stranded cDNA molecule for each complementary

RNA. Finally, the reverse transcription reaction was ceased by inactivation of reverse transcriptase enzymes at 85°C for 5 minutes. The reverse-transcription cDNA products were stored at –20 °C.

2.3.4 Primer design

Target-specific PCR primers were designed to match the most frequent coding region between different splice variants of *BCAT1* and *IKZF1*. Ensembl database (e!Ensemble 2016) was used to check the appropriate flanking regions of target genes. Primers sets were subjected to the Netprimer software (PREMIER Biosoft 2016) to check for the presence of self-complementarity regions, hair-pin structures and cross dimers regions, and to examine other important primers parameters including primer length, GC content, melting temperature and GC clamp (Table 6). Finally, primers pairs were searched again in the publicly accessible genomic database by using primer-BLAST (NCBI 2016) to examine their specificity to the target genes and display the other potential targets. The primers then were obtained from Geneworks Pty Ltd.

Table 6. The nucleotide sequences and features of *BCAT1* and *IKZF1* gene primers.

	BCAT1		***IKZF1***	
	Forward	**Reverse**	**Forward**	**Reverse**
Sequences (5' to 3')	GGTCCCATATTCA ACATCTGC	CACCTTTCCAGGCT CTTACA	GTCTCATCTACCT GACCAACCAC	TTGTACACCTTCAT CTGCTCC
Primer length	21	20	23	21
Tm (netprimer)	56.86	55.29	57.71	55.05
Net primer score	92	99	100	86
GC Clamp	2	1	2	2
GC%	47.62	50	52.17	47.62
Self-Dimer (ΔG)	-3.91	None	None	-7.55
Hairpin (ΔG)	None	-0.17	None	None
Repeats (#of pairs)	None	None	None	None
Run (#of bases)	3	3	None	None
Genomic Position		4th /5th exon		6th exon
Primer dimer (ΔG)		-4.55		None
Amplicon length (bp)		197		160

Another set of primers with different genomic position (2nd/3rd Exons) for amplifying *IKZF1* gene were taken from published research (Hu et al. 2011). Primers sets designed to amplify reference genes (*HPRT1, B2M* and *ACTB*) were obtained from difference sources. Sequences of forward and reverse primers for amplifying *HPRT1* and *B2M* genes were taken from published research (Cicinnati et al. 2008) and ordered (together with the *IKZF1* published primers) from Geneworks Pty Ltd (Table 7). Primers were excluded from further use if there was: (1) amplification in the no reverse transcription wells, indicating interference by gDNA; (2) amplification in the no template control wells, indicating formation of primer dimers; and/or (3) the melt curve was not giving a single peak controls suggesting the presence of nonspecific binding or gDNA contamination.

The three primers sets selected for use were the self-designed *BCAT1* and the published *HPRT1* and *IKZF1* sequences. Efficiency of the selected primers were assessed by running PCR of a standard curve created from different series of dilutions (43.1 ng/µL, 14.3 ng/µL, 4.7 ng/µL, 1.5 ng/µL, and 0.5 ng/µL) of cDNA from a solution of pooled para-tumour tissues. Each concentration was assessed in triplicate.

Table 7. The nucleotide sequences of published primers used for PCR amplification and the sizes of the PCR products.

Gene	Primer	Sequence (5' to 3')	Amplicon length (bp)	Genomic position	Publisher
IKZF1	Forward	GCAAAGCTCCAAGAGTGACAGA	80	2nd/3rd Exons	(Hu et al. 2011)
	Reverse	AGGCACGCCCATTCTCTTC			
HPRT1	Forward	TGACACTGGCAAAACAATGCA	94	6th Exon	(Cicinnati et al. 2008)
	Reverse	GGTCCTTTTCACCAGCAAGCT			
B2M	Forward	CTCCGTGGCCTTAGCTGTG	69	1st Exon	(Cicinnati et al. 2008)
	Reverse	TTTGGAGTACGCTGGATAGCCT			
ACTB		Not provided			Qiagen

3.3.4 Agarose gel Electrophoresis

Primer product specificity for the self-designed *BCAT1* was confirmed through agarose gel electrophoresis. Agarose gel (0.02%) was made using 0.7 g of Low EEO agarose (Bioline, Alexandria, NSW, Australia) in 35 mL of 1x TAE buffer (Thermo Fisher Scientific) and heated until dissolved using a microwave. Ethidium bromide (Sigma-Aldrich, Australia) was used as staining dye. The gel was placed in the horizontal gel apparatus (Bio-Rad) containing 1x TAE buffer and run until the loading dye (Gel Loading Dye Blue 6x, Bio-Red) was no more than 2/3 the way down the gel at ~50 volts. DNA was visualized and examined at 380 nm using the Gel Doc™ EZ Gel Documentation System (Bio-Rad). BioLabs 100 bp DNA ladder was used to determine the size of target DNA band. A total volume of 6 µL containing 5µl of *BCAT1* PCR product and 1µl of loading dye was loaded in lane in the gel.

2.3.5 Real time RT-PCR

The quantification of *BCAT1* and *IKZF1* relative to a reference gene (*HPRT1*) was carried out using a QuantiNova SYBR Green PCR Kit (Catalog number 208052, Qiagen GmbH, Hilden, Germany) and real-time PCR (Rotor-Gene® Q instrument). RT-PCR amplification experiments were performed in triplicate in a final PCR volume of 10 µL. All of the primers were assessed in separate wells. The amplification mixtures of real time RT-PCR contained 5 µL of QuantiNova SYBR Green PCR Master Mix (QuantiNova DNA *Taq* Polymerase inactivated by QuantiNova Antibody, QuantiNova SYBR Green PCR Buffer, dNTP mix and SYBR Green I), 2 µL of forward and reverse primers of either *BCAT1*, *IKZF1* or *HPRT1* (concentration = 7 nmol) and 3 µL of 1:100 diluted cDNA template (of concentration approximate 25 ng/ µL). The cycling conditions comprised of 2 min 95ºC for activation of polymerase and 50 cycles: 5 second at 95°C for denaturation and 10 seconds at 60°C for combined annealing/extension. The data was acquired

during the combined annealing/extension step of cycle on the green channel. The amplification efficiency was checked at the end of each PCR run by creating a melting curve. The start and end temperatures of melt curve were 55°C and 95°C respectively, the temperature was increased by increments of 1ºC, waiting for 5 seconds before each acquisition. Each RT-PCR assay involved two different controls tested in triplicate; a no template control (RNase free water) and a no reverse transcriptase control (a sample of pooled RNA).

2.3.6 Gene expression quantification

Gene expression results were analyzed using Rotor-Gene Q Series Software (version 2.0.2). The expression levels of target genes (*BCAT1* or *IKZF1*) were normalized against the expression level of *HPRT1* gene. Normalization was performed using *Q-Gene* application that takes into consideration the differences in PCR amplifications efficiencies (E) between target genes and the reference gene (Simon 2003). The relative expression of *BCAT1* and *IKZF1* were expressed as the Mean normalized expression (MNE) where MNE equals to $(E_{HPRT1})^{\text{mean Ct of } HPRT1}/(E_{BCAT1 \text{ or } IKZF1})^{\text{mean Ct of } BCAT1 \text{ or } IKZF1}$. The take-off point which is equivalent to the Ct value was used for results analysis. The take-off point is 20 % of the peak that represents the maximum rate of exponential amplification (McCurdy & McGrath 2008).

2.4 Statistical analysis

Statistical analyses were performed using GraphPad Prism, version 6.05 (GraphPad Software, Inc). Normality of the data were tested with D Agostino-Pearson amnibus normality test. As the data was shown to have non-parametric distribution, comparison between two groups were performed with Mann-Whitney test (two-tailed) and comparison between more than two groups were performed using Kruskal Wallis test. Correlations between data were assessed with a Spearman

correlation rank test. Chi-squared analysis was used for comparing the proportions of samples with significant methylation (≥5%) between groups, Yates correction was used when any sample size in the comparisons was less than 5. P-values of less than 0.05 were considered statistically significant.

Chapter 3. Results

3.1 Methylation level results

3.1.1 DNA concentration

DNA was extracted from 100 tissue samples, a tumour and para-tumour tissue sample from each CRC patient. The concentration of DNA ranged between 20-180 ng/µL depending on the size of the tissue sample used for extraction. No significant difference was observed in DNA concentration between tumour and para-tumour tissue samples (para-tumour median DNA concentration = 125.7 ng/µL; tumour median DNA concentration = 135.2 ng/µL; P= 0.915).

3.1.2 Development of the methylation PCR assay

The methylation levels of target genes (*BCAT1* and *IKZF1*) in tumour and para-tumour tissue samples were read from external standard curves generated using a dilution series of methylated Jurkat gDNA. Correlation R-values for *ACTB* gene standard curves ranged from 0.96-0.99, for *BCAT1* gene standard curves ranged from 0.91-0.98, and for *IKZF1* gene standard curves ranged from 0.94-0.98. The results of a typical standard curve and PCR amplification curve for *ACTB* are shown in Figure 3.

A B

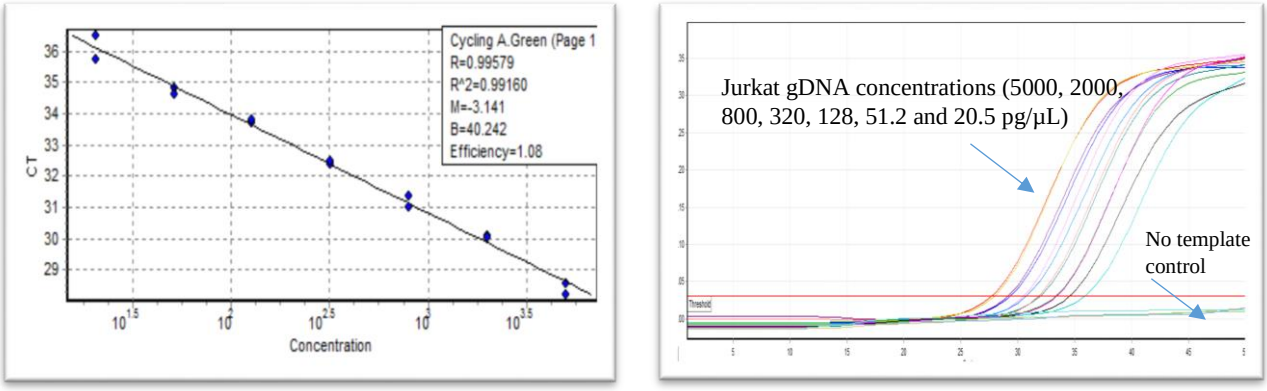

Figure 3.A) A typical standard curve of cycle threshold Ct for *ACTB* expression against the concentration of bisulfite-converted Jurkat gDNA. Serial dilutions of bisulfite-converted Jurkat gDNA (5000, 2000, 800, 320, 128, 51.2 and 20.5 pg/µL) were quantified in duplicate by quantitative real-time PCR. Some duplicates are not evident due to overlapping. **B)** Real-time PCR amplification of *ACTB* gene found in bisulfite-converted Jurkat gDNA serial dilutions. Amplification was indicated by an increasing fluorescence of HEX fluorophore of *ACTB* gene probe on the y-axis. No fluorescent signal was obtained from the no template control. The threshold was set just above the no template control.

3.1.3 Methylation levels of tumour tissues compared with para-tumour tissues

To study differences in the levels of methylated *BCAT1* and *IKZF1* between tumour and para-tumour tissues, changes were analysed based on results of the 36 patients who had no neoadjuvant treatment prior to surgery (Table 3. See Appendix A for the individual patient results used for methylation analysis). The methylation results of *BCAT1* and *IKZF1* (expressed as a % of total *ACTB*) in colorectal tumour tissue compared with adjacent para-tumour tissue samples of CRC patients showed significant differences, with significantly higher methylation levels in the tumour tissue (Figure 4). In the colorectal tumour tissue the median levels of methylated *BCAT1* and *IKZF1* were 39.8% and 53.3% respectively, in contrast the median levels of methylation in the adjacent para-tumour tissues were 3.2% and 0% for *BCAT1* and *IKZF1* respectively (P = 0.001).

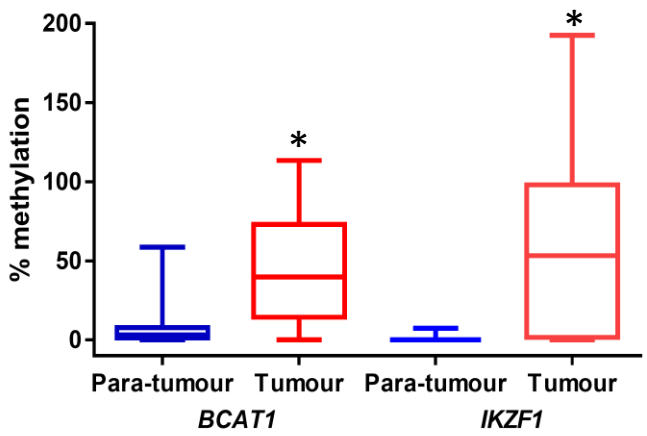

Figure 4. Methylation status of *BCAT1* and *IKZF1* in colorectal tumour tissues and para-tumour tissues. The two genes (*BCAT1* and *IKZF1*) in tumour (n = 36) and corresponding para-tumour tissues (n = 36) are plotted on the x-axis, and the percentage of methylation of each gene (*BCAT1* and *IKZF1*) 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. *P < 0.05 compared with para-tumour results.

When results were considered as patients that either had methylation absent (methylation < 5%) or present (methylation ≥ 5%), it was found that 91% of colorectal tumour tissues (n = 36) had *BCAT1* methylated while only 30% of para-tumour tissues had *BCAT1* methylated (P < 0.001; Figure 5). Similarly, it was found that 71% of tumour tissues had *IKZF1* methylated while only 2% of para-tumour tissues had *IKZF1* methylated (P < 0.001, Figure 5).

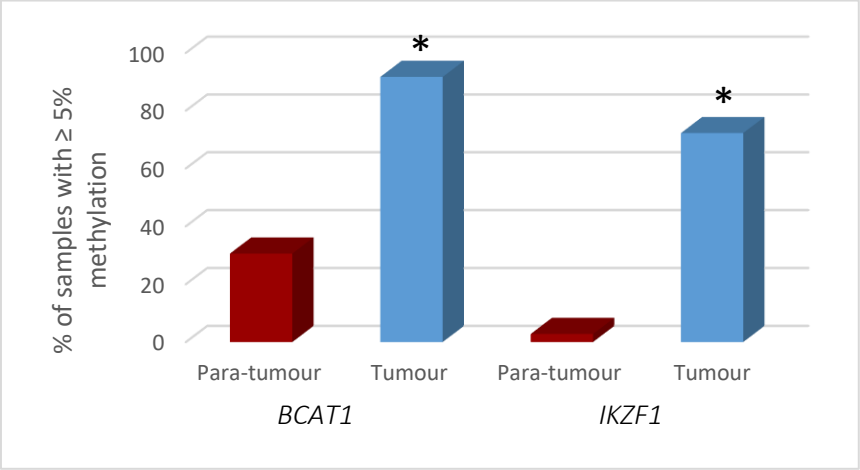

Figure 5. The proportions of tumour and para-tumour tissues with methylated *BCAT1* and *IKZF1*. Colorectal tumour tissues are in blue (n = 36) and the matching para-tumour tissues are in red (n = 36). Samples were considered to have significant levels of methylation when levels were ≥ 5%. *represents P<0.05 compared to para-tumour results.

The effects of field cancerization of methylated *BCAT1* and *IKZF1* were examined by assessing methylation in the tissue collected close to the tumour (distance ≤ 50 mm of tumour; n = 15), and distant to the tumour (distance > 50 mm of tumour; n = 20). One sample collection distance was not recorded and was excluded from this analysis. No significant effects of levels of methylated *BCAT1* and *IKZF1* of the surrounding (adjacent and distance) para-tumour tissues were observed (close to tumour median = 34.2% and 25.5%; distant to the tumour median = 38.9% and 58.5%; P = 0.988 and 0.913 respectively). This was further confirmed through the lack of correlations between the distance where para-tumour tissues were collected and levels of methylated *BCAT1* (R = -0.215; P = 0.215) and *IKZF1* (R = -0.118; P = 0.499).

3.1.4 The effects of clinical and tumour features on tissue methylation levels

The effects of clinical factors, including gender, age and tumour site on levels of methylated *BCAT1* and *IKZF1* were also evaluated. Of the group without neoadjuvant therapy (n = 36), 52%

were females and 48% were males. No statistical difference was found in *BCAT1* and *IKZF1* methylation levels based on gender in both tumour and para-tumour tissues (Table 8 and 9). While there were higher median levels of *BCAT1* and *IKZF1* methylation observed in female tumour tissue (51% and 62% respectively) compared to male median levels (35% and 27.5%), this did not reach statistical significance (P = 0.311 and 0.323 respectively).

The CRC patients (n = 36) could be divided into two age groups; group 1 (n=22) age ≤ 65 years, group 2 (n = 14) age > 65 years. There were no significant age related changes in the levels of methylated *BCAT1* and *IKZF1* between the two age groups in tumour tissues (group 1 median = 35.8% and 61.25%; group 2 median = 44.8% and 47.8%; P = 0.804 and 0.493, Table 8 and 9). In para-tumour tissues, the methylated *BCAT1* was slightly higher in the younger age group (median = 7.9%) compared with the older age group (median = 1.9%; P = 0.555), but this did not reach statistical significance, while, in both age groups, no methylated *IKZF1* was observed (both medians = 0%).

About two thirds (69%) of tumours of non-treated CRC patients (n = 36) were located in the colon, including transverse, right and left colon, and 31% of tumours were resected from the rectum. There were no significant location-related changes in the levels of methylated *BCAT1* and *IKZF1* in tumour and para-tumour tissues (Table 8 and 9).

Table 8. Association of levels of methylated *BCAT1* and colorectal tumour clinical parameters.

		Tumour		Para-tumour	
Clinical parameters	N = 36	Median methylated *BCAT1* (25^{th}-75^{th} percentiles)	P-value	Median methylated *BCAT1* (25^{th}-75^{th} percentiles)	P-value
Gender					
Male	17	35.0 (13.4-53.3)		3.2 (1.3-4.9)	
Female	19	51.1 (21.1-77.5)	0.311	3.1 (1.5-9.1)	0.669
Age (year)					
≤ 65	22	35.8 (13.4-75.7)		1.9 (1.2-3.5)	
> 65	14	44.8 (30.9-64.7)	0.804	7.4 (3.9-11.8)	0.555
Location					
Colon	25	51.1 (25.2-81.9)		2.1 (1.2-4.7)	
Rectum	11	30.6 (12-47.2)	0.106	6.8 (2.15-10.9)	0.126
Lymphatic invasion					
Yes	15	35.0 (13.8-62.9)		2.1 (1.2-9.7)	
No	21	46.8 (24.9-76.6)	0.360	3.8 (1.3-6.3)	0.961
Distal metastasis					
Yes	5	35.0 (0.8-36.6)		1.0 (0.8-2.1)	
No	31	46.8 (20.9-74.8)	0.245	3.8 (1.7-8.1)	0.033

Table 9. Association of levels of methylated *IKZF1* and colorectal tumour clinical parameters.

		Tumour		Para-tumour	
Clinical parameters	N = 36	Median methylated *IKZF1* (25^{th}-75^{th} percentiles)	P-value	Median methylated *IKZF1* (25^{th}-75^{th} percentiles)	P-value
Gender					
Male	17	27.5 (0.8-87.3)		0 (0-0)	
Female	19	62.0 (36.3-99.8)	0.323	0 (0-0)	0.712
Age (year)					
≤ 65	22	61.2 (7.4-109.7)		0 (0-0)	
> 65	14	47.8 (6.3-78.3)	0.493	0 (0-0.2)	0.043
Location					
Colon	25	60.5 (25.5-83.6)		0 (0-0)	
Rectum	11	45.6 (0-124.7)	0.912	0 (0-0)	0.523
Lymphatic invasion					
Yes	15	41.2 (0.4-68.8)		0 (0-0)	
No	21	60.5 (12.1-116.1)	0.402	0 (0-0)	0.364
Distal metastasis					
Yes	5	36.7 (27.5-62.0)		0 (0-0)	
No	31	56.5 (2.2-94.5)	0.989	0 (0-0)	0.889

The relationship between methylated *BCAT1* and *IKZF1* in colorectal tumour samples (n = 36) was assessed. A significant positive correlation between the levels of methylated *BCAT1* and *IKZF1* in colorectal tumour tissues, (R = 0.486; P = 0.003) was observed (Figure 6).

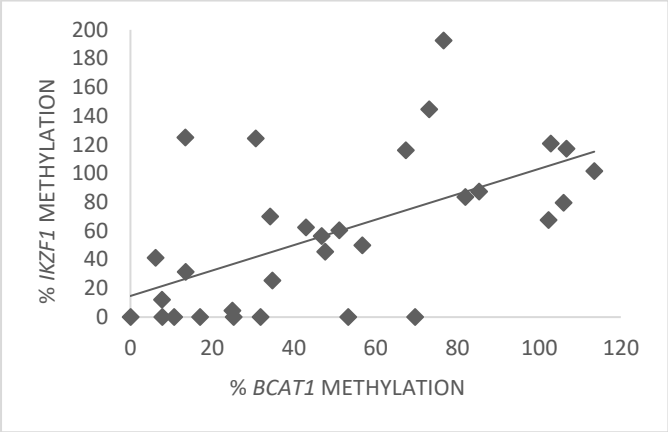

Figure 6. The correlation between the levels of methylated *BCAT1* and *IKZF1* in colorectal tumour tissues. The values of levels of methylated *BCAT1* and *IKZF1* in colorecal tumour tissues (n = 36) were plotted on (y) and (x) axes respectevely P = 0.003.

The CRC patients were diagnosed with different stages of the illness (n = 8 stage I; 12 stage II; 11 stage III; 5 stage IV, Table 3). The influence of developmental stages of CRC on levels of methylated *BCAT1* and *IKZF1* was assessed (Figure 7). While stage IV colorectal tumours had the lowest median methylation levels for both *BCAT1* and *IKZF1*, statistical analysis showed no overall differences as the tumour stage progressed (*IKZF1* median = 61.5%, 68.1%, 45.6% and 36.7% for stage I, II, III and IV respectively; P = 0.554; *BCAT1* median = 47%, 40.7%, 47.6% and 35% for stage I, II, III and IV respectively; P = 0.734).

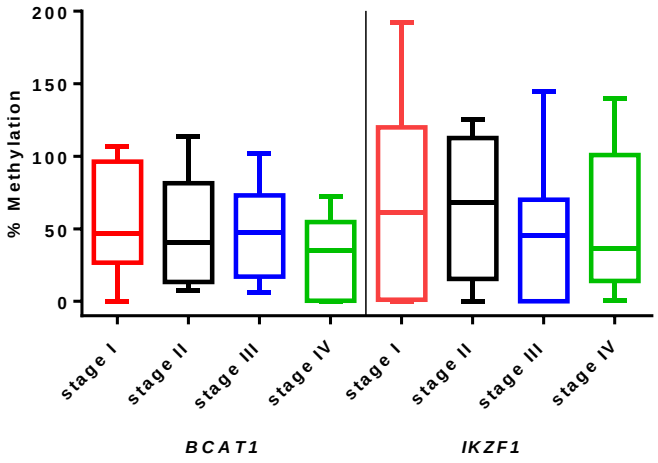

Figure 7. CRC stage-related methylation levels of *BCAT1* and *IKZF1*. Levels of methylated *BCAT1* and *IKZF1* in relation to colorectal cancer stage (TNM staging). Different colorectal cancer stages: stage I (n = 8), II (n = 12), III (n = 11) and IV (n = 5), are plotted on the x-axis, and the percentage of methylation of each gene (*BCAT1* and *IKZF1*) are plotted on the y-axis. Data are expressed as median, 25th and 75th percentiles within the box, with bars showing the minimum to maximum.

Of the 36 patients who had no treatment prior to surgery, 58% (n = 21) had been diagnosed with invaded lymph nodes, and 21% (n = 5) had metastases (Table 3). The relationships between levels of methylated *BCAT1* and *IKZF1* and the presence of metastases and lymph node invasion were assessed. There were no associations between levels of methylated *BCAT1* and *IKZF1* and the presence of metastases and lymph node invasions in tumour and para-tumour tissues (Table 8, 9). The degree of gut layers' invasion (T-stages) by tumour was found to vary among the 36 patients. There were 11% (n = 4), 19% (n = 7), 44% (n = 16) and 25% (n = 9) of patients diagnosed with stage T1, T2, T3, and T4 respectively (Table 3). The relationships between levels of methylated *BCAT1* and *IKZF1* and the degree of tumour invasiveness through the layers of gut were evaluated. A negative, but not significant, trend was observed between T- stage and levels of methylated

BCAT1 (median = 56%, 51.1%, 40.9% and 34.2% for T1, T2, T3 and T4 respectively; P = 0.851; Figure 8). The levels of methylated *IKZF1* did not differ between the different T stages (median = 52.2%, 62.5%, 53.2% and 27.5% for T1, T2, T3 and T4 respectively; P = 0.732; Figure 8).

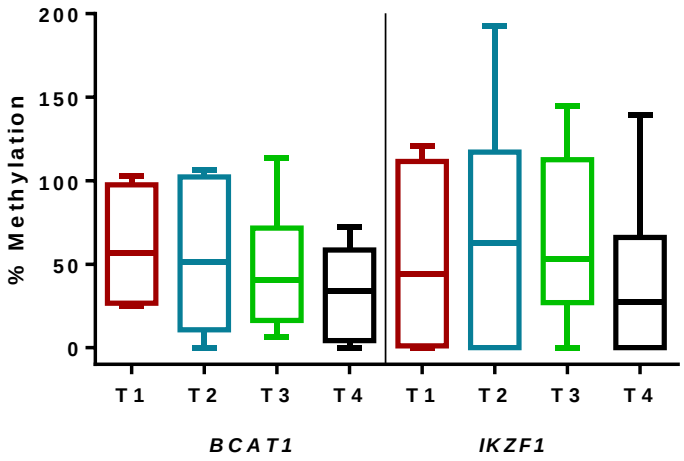

Figure 8. Levels of methylated *BCAT1* and *IKZF1* in relation to colorectal cancer T-stage (gut layers' invasiveness). Different T-stages (T1 n = 4, T2 n = 7, T3 n = 16, and T4 n = 9) of colorectal tumours are plotted on the x-axis, and the percentage of methylation of each gene (*BCAT1* and *IKZF1*) 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.

3.1.5 The effects of neoadjuvant therapy on methylation levels

Out of the 50 CRC patients, 14 participants were subjected to neo-adjuvant treatments, either radiotherapy (n =4) or radiotherapy and chemotherapy (n =11), prior to surgical resection (Table 3 and 10). Levels of methylation were not dependent on location of tumour (rectal versus colon, Table 8 and 9), and therefore all tumours from patients that had not received neoadjuvant therapy could be compared to the rectal tumours that had undergone chemotherapy and/or radiation treatment. The effects of neoadjuvant therapy on the levels of methylated *BCAT1* and *IKZF1* in

colorectal tumour tissues compared with non-treated colorectal tumour tissues showed significant decreases. There were no significant differences in the levels of methylated *BCAT1* and *IKZF1* between the 65% of tumour tissues subjected to chemotherapy and radiotherapy (both medians = 0%), and the 29% of tumours that received radiotherapy as the only pre-surgical treatment (median = 1.9%; 2.3%; P = 0.218 and 0.203). The high levels of methylated *BCAT1* and *IKZF1* found in the non-treated colorectal tumour tissues (median = 39.75% and 53.25%) were reduced by neoadjuvant therapy. Median levels of methylated *BCAT1* and *IKZF1* in the treated tumour tissue were both 0.5%, statistically lower than non-treated levels (P = 0.010, Figure 9). No significant neoadjuvant (radiation)-induced bystander effects were observed on the levels of methylated *BCAT1* and *IKZF1* in the treated para-tumour tissues (median =7.7% and 0%) compared with these levels in non-treated para-tumour tissues (median= 3.14% and 0%; P= 0.167 and 0.261, Figure 9).

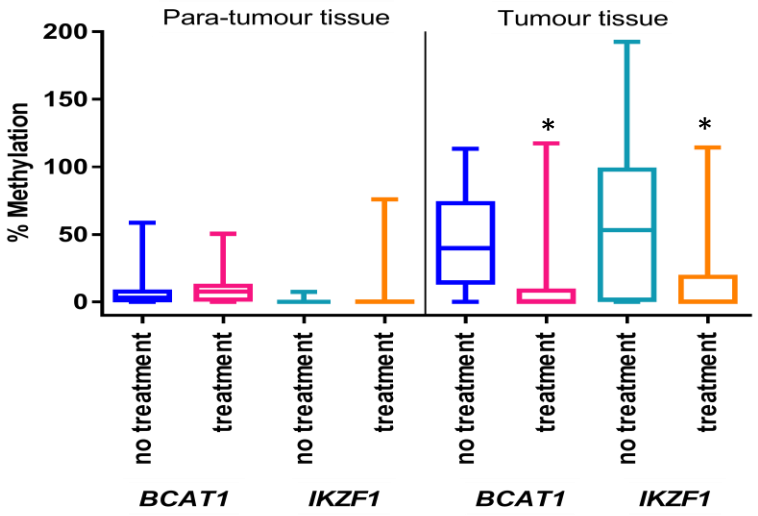

Figure 9. The effects of neoadjuvant therapy on the levels of methylated *BCAT1* and *IKZF1* in colorectal tumour and para-tumour tissues. The two genes (*BCAT1* and *IKZF1*) in colorectal tumour tissues and para-tumour tissues (both treated; n = 14 and non-treated; n = 36) are plotted on the x-axis, 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. *represents P< 0.05 compared to non-treated tumour tissues.

When results were considered as patients that either had methylation absent (methylation < 5%) or present (methylation ≥ 5%), it was found that with no neoadjuvant therapy 71% of tumour tissues (n = 36) had *IKZF1* methylated while with neoadjuvant therapy only 21.4% of tumour tissues (n = 14) had *IKZF1* methylated (P <0.001, Figure 10). Also, it was found that with no neoadjuvant therapy 91% of tumour tissues had *BCAT1* methylated while with neoadjuvant therapy only 28.5% of tumour tissues had *BCAT1* methylated (P 0.04, Figure 10).

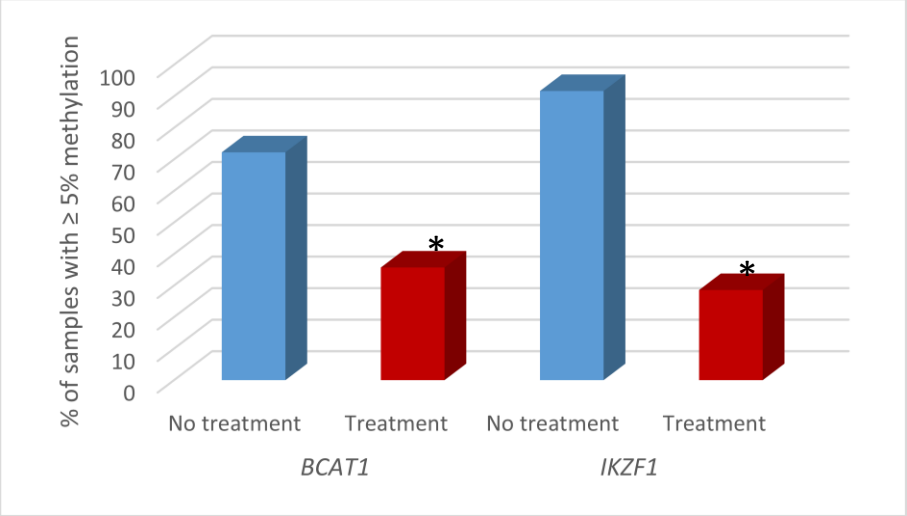

Figure 10. The effects of neoadjuvant therapy on the proportions of colorectal tumours with methylated *BCAT1* and *IKZF1*. Proportion of samples with methylated *BCAT1* and *IKZF1* in colorectal tumour tissues with (n = 14) and without (n = 36) neoadjuvant therapy. Samples with ≥ 5% methylation (either *BCAT1* or *IKZF1*) were considered to be positive for methylation. *represents P< 0.05 compared to non-treated tumour tissues.

The colorectal tumours subjected to neoadjuvant therapy could be divided into two groups based on tumours' responses to treatments measured by the percentage of size shrinkage; a low response was when tumours had shrunk less than 50% of their original size (n = 5), and a high response was when tumours had shrunk more than 50% of their original size (n = 7). Two tumours did not have

pre-treatment size and had to be excluded from this analysis (Table 10). The levels of methylated *BCAT1* and *IKZF1* within the tumour were associated with the response to neoadjuvant therapy, with significantly lower levels in tumours that had shrunk to 50% or less of the original size (both medians = 0%), compared to tumours with less size reduction (median = 6.1% and 5.3%, P = 0.019 and 0.045, Figure 11).

Table 10. List of radiation dose received by 14 patients, and their effects on size and stage of tumours.

		Pre-treatment tumour		Post-treatment tumour		
ID	**Radiotherapy total (Gy)**	**Size (mm)**	**Stage**	**Size (mm)**	**Stage**	**Tumour % of original size**
28	50.4	61	IVA	1.5	IVA	2.5
3474	50	70	IVA	20	IVA	28.6
560	50.4	61	IIIC	18	IIIB	29.5
822	50.4	85	IIIC	30	I	35.3
1811	50.4	75	IIA	36	I	48.0
926	50.4	62	IIIB	30	IIIA	48.4
965	50.4	80	IIIB	40	IIIB	50.0
2298	50.4	76	IIIB	40	IIIB	52.6
1010	25	37	IIIB	25	IIA	67.6
1411	50.4	36	IVA	25	IVA	69.4
708	50.4	80	IIIB	60	I	75.0
3418	50	70	IIIB	80	IIA	114.3
3024	50.4	Unknown	IIIB	20	IIIB	Unknown
3153	50.4	Unknown	IIIB	35	unknown	Unknown

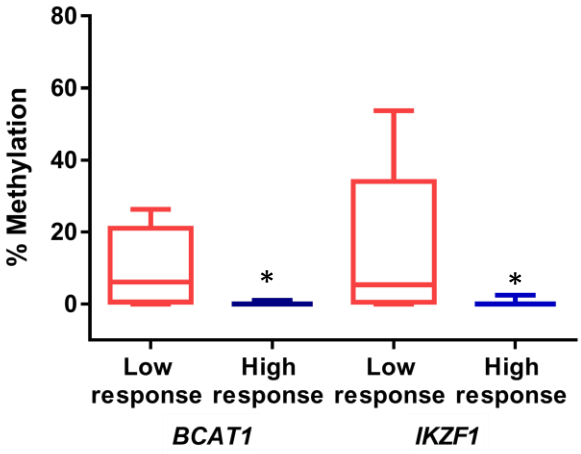

Figure 11. Levels of methylated *BCAT1* and *IKZF1* in tumours with different responses to neoadjuvant therapy. Colorectal tumours with low response to neoadjuvant therapy (where tumours shrunk <50% of the original size; n = 5) and colorectal tumours with high response to neoadjuvant therapy (where tumours shrunk ≥ 50% of the original size; n = 7) are plotted on the x-axis. The percentages of methylation levels of *BCAT1* and *IKZF1* were plotted on the y-axis. Data are expressed as median, 25th and 75th percentiles within the box, with bars showing the minimum to maximum. *represents P< 0.05 compared to low response tumour tissues.

3.2 Gene expression results

3.2.1 RNA concentration

RNA was extracted from 100 tissue samples, a tumour and para-tumour tissue sample from each CRC patient. The concentration of RNA ranged between 24-1818 ng/µL depending on the size of the tissue sample used for extraction. No significant difference was observed in RNA concentration between tumour and para-tumour tissue samples (para-tumour median RNA concentration = 318.6 ng/µL; tumour median RNA concertation = 468.1 ng/µL; P= 0.815).

3.2.2 Optimisation of the gene expression real-time PCR assay

Assessment to establish the optimal cDNA concentration for reverse transcription real time PCR showed that the best amplification results were obtained by using 60-75ng of cDNA. Melt-curve analysis was performed to identify the PCR amplification specificity for each gene (*BCAT1*, *IKZF1* and *HPRT1*). This analysis generated a curve with single peak determined by product specific melting temperature (Figure 12).

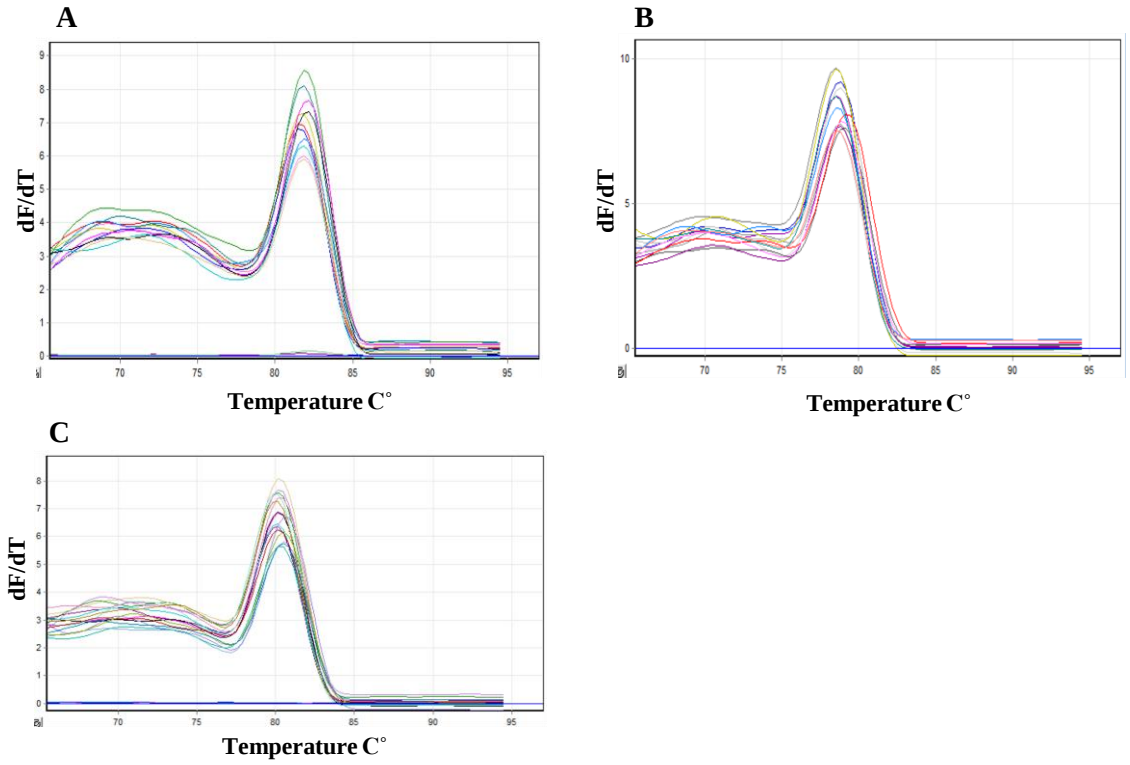

Figure 12. Melt curve plots of amplified *BCAT1*, *IKZF1* and *HPRT1* genes. Melt curves generated by Rotor-Gene® Q instrument (Qiagen) representing products from the amplifications of (A) *BCAT1*, (B) *IKZF1* and (C) *HPRT1* (temperature ranges 65-95C). The rate of change of fluorescence in the reaction (dF\dT) is plotted on the y-axis and the temperature change is plotted on the x-axis. The real-time change of fluorescence is the function of temperature. The single peak represents the amplification of one product by PCR.

In addition, the self-designed primers for *BCAT1* had product confirmation through running an agarose gel. A clear DNA band of size of 197 bp was observed in lane 2 indicating the amplification of *BCAT1* via the self-designed primer set (Figure 13).

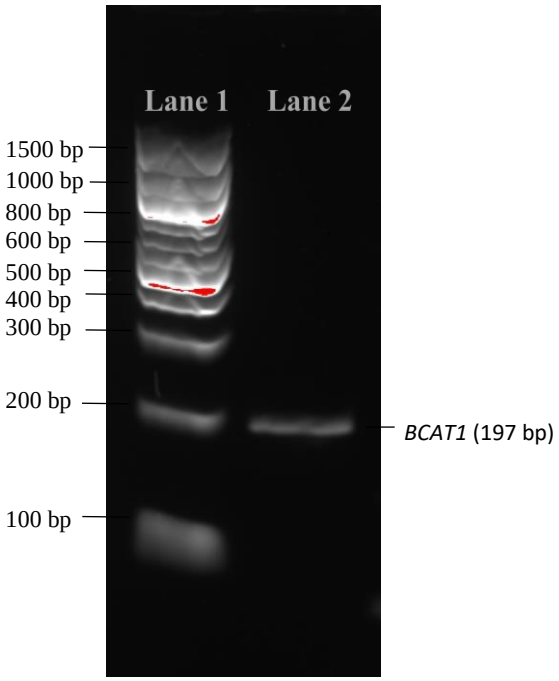

Figure 13. Results of agarose gel electrophoresis of the product from the *BCAT1* gene primers. Gel (0.2%) was stained with ethidium bromide dye and examined by Bio-Rad EZ imager. Lane 1: 1kb BioLabs 100 bp DNA ladder (1µl ladder and 5µl of RNase free water) and lane 2: amplification *BCAT1* gene (5µl of *BCAT1* PCR product and 1µl of loading dye).

The efficiencies for the primers sets of *BCAT1*, *IKZF1* and *HPRT1* were 1.791, 1.874 and 1.855 respectively (Figure 14). The different efficiencies were taken into account with the gene expression calculations, using the normalized mean expression equation (Krakowczyk et al. 2010).

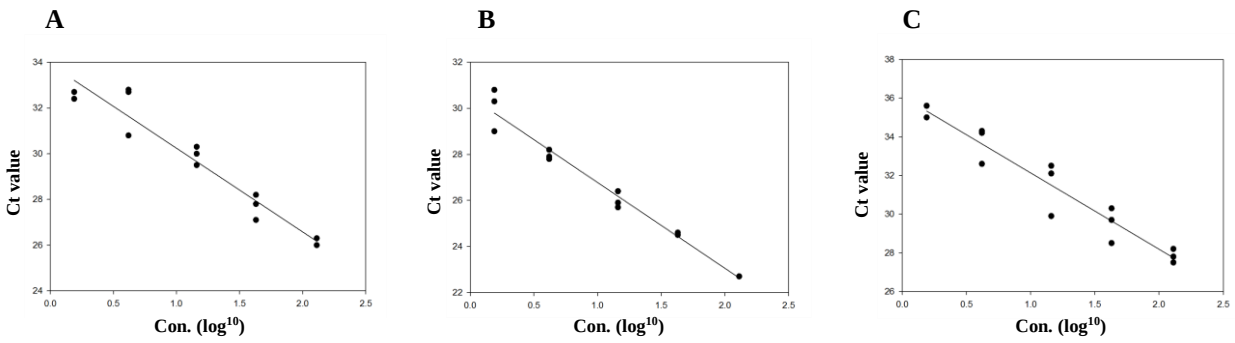

Figure 14. Real-time PCR standard curves for assessing efficiency of different primer sets. Efficiency standard curves for (A) *BCAT1* primers set, (B) *IKZF1* primers set and (C) *HPRT1* primers set were made using SigmaPlot for Windows Version 11.0 (Systat Software, Inc. Germany). The standard curves were created by running PCR of serial dilutions of pooled cDNA of para-tumour tissues. The log of concentrations of cDNA were plotted on the x-axis and the Ct (take-off points) values were plotted on y-axis.

3.2.3 Gene expression levels of colorectal tumour and para-tumour tissues

To study differences in the levels of *BCAT1* and *IKZF1* gene expression between tumour and para-tumour tissues, as done for methylation results, changes were analysed based on results of 36 patients who had no treatment prior to surgery (Table 3. See Appendix B for the individual patients results of *BCAT1, IKZF1* and *HPRT1* used for gene expression analysis). Two para-tumour tissues were excluded from analysis because there was no amplification in their reference genes indicating poor quality RNA. The results of *BCAT1* and *IKZF1* gene expression in colorectal tumour tissues (n =36) compared with adjacent para-tumour tissue samples (n =34) are shown in Figure 15. The levels of *IKZF1* relative expression observed in colorectal tumours (median = 0.030) was significantly lower compared with the expression of the adjacent para-tumour tissues (median = 0.069; P = 0.001). There were no significant differences found between *BCAT1* expression in colorectal tumour tissues (median = 0.077) and levels in the adjacent para-tumour tissues (median = 0.125; P = 0.799).

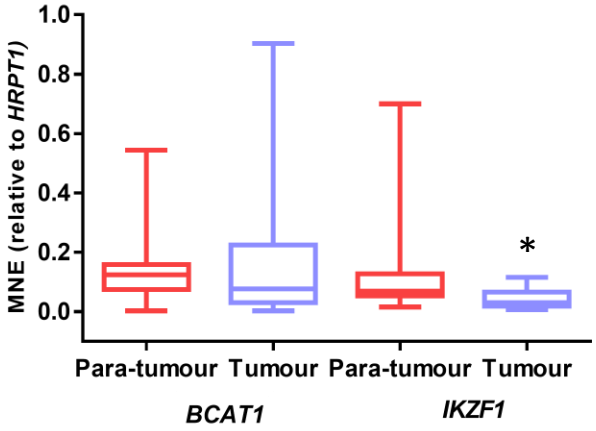

Figure 15. The relative gene expression of *BCAT1* and *IKZF1* in colorectal tumour and para-tumour tissues. Tumour tissues (blue; n = 36) and the matching para-tumour tissue (red; n = 34) are plotted on the x-axis, and the means of normalized genes expression levels are plotted on y-axis. The gene expression results are normalized to the levels of *HPRT1* gene expression in corresponding samples. Data are expressed as median, 25th and 75th percentiles within the box, with bars showing the minimum to maximum. *represents P< 0.05 compared to para-tumour results.

The influence of stages of CRC on levels of *BCAT1* and *IKZF1* gene expression was assessed (Figure 16). As the cancer stage progressed there were no significant differences in the levels of *IKZF1* gene expression (median = 0.029, 0.35, 0.039, and 0.055 for stage I, II, III and IV respectively; P = 0.454), or for levels of *BCAT1* gene expression (median = 0.042, 0.151, 0.077 and 0.195 for stage I, II, III and IV respectively; P = 0.384).

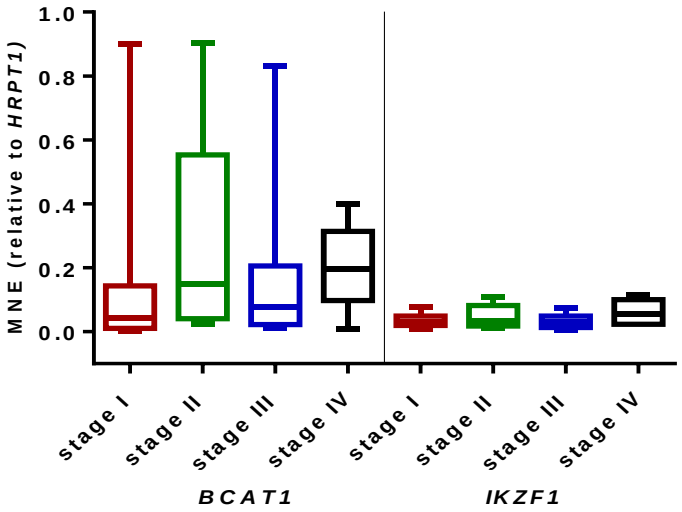

Figure 16. Stage-related gene expression levels of *BCAT1* and *IKZF1* in colorectal tumour tissues. The relative gene expression levels of *BCAT1* and *IKZF1* in relation to colorectal cancer stage (TNM staging). Different colorectal cancer stages: stage I (n = 8), II (n = 12), III (n = 11) and IV (n = 5), are plotted on the x-axis, and the means of normalized genes expression levels were plotted on y-axis. The gene expression results are normalized to the levels of *HPRT1* gene expression in corresponding samples. Data are expressed as median, 25th and 75th percentiles within the box, with bars showing the minimum to maximum.

3.2.4 The effects of neoadjuvant therapy on gene expression

The effects of neoadjuvant therapy on the levels of *BCAT1* and *IKZF1* gene expression in colorectal tumour tissues were assessed. Colorectal tumours that had been treated with neoadjuvant therapy had a significantly higher expression of *IKZF1* compared with non-treated tumours (treated = 0.10; non-treated = 0.03, P = 0.001). Similarly, there was a significant difference in *BCAT1* expression between treated and non-treated tumour tissues (non-treated = 0.07; treated = 0.35, P = 0.012; Figure 17).

There were no significant differences in the levels of *BCAT1* and *IKZF1* gene expression between the 65% of tumour tissues subjected to chemotherapy and radiotherapy (median = 0.42; 0.13), and the 29% of tumour that received radiotherapy as the only treatment (median = 0.19; 0.06; P = 0.718 and 0.609).

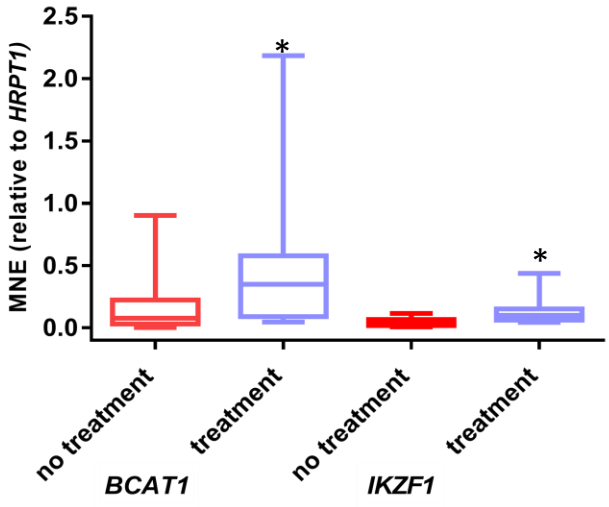

Figure 17. The effects of neoadjuvant therapy on the relative levels of *BCAT1* and *IKZF1* gene expression in colorectal tumour tissues. The two genes (*BCAT1* and *IKZF1*) in treated (n = 14) and non-treated (n = 36) colorectal tumour tissues are plotted on the x-axis, and the means of normalized genes expressions levels are plotted on y-axis. The gene expression results are normalized to the levels of *HPRT1* gene expression in corresponding samples. Data are expressed as median, 25th and 75th percentiles within the box, with bars showing the minimum to maximum. *P < 0.001 compared to non-treated tissue

There were also no differences in the *BCAT1* and *IKZF1* relative expression levels with tumours that had the greatest response to therapy (≥50% tumour size reduction) compared to the tumours with the low response to therapy (<50% tumour size reduction; Figure 18). Median of *BCAT1* and *IKZF1* gene expression of high response tumours were 0.092 and 0.044 and low response tumours were 0.151 and 0.048; P = 0.317 and 0.654 respectively.

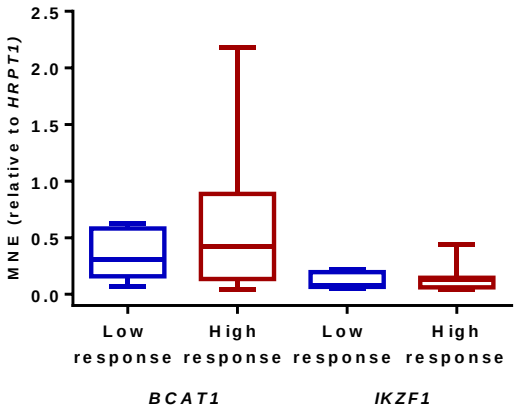

Figure 18. The relative levels of *BCAT1* and *IKZF1* gene expression in colorectal tumours with different responses to neoadjuvant therapy. Colorectal tumours with low response to neoadjuvant therapy (where tumours shrunk <50% of the original size; n = 5) and colorectal tumours with high response to neoadjuvant therapy (where tumours shrunk ≥ 50% of the original size; n = 7) are plotted on the x-axis, and the gene expressions levels are plotted on the y-axis. The gene expression results are normalized to the levels of *HPRT1* gene. Data are expressed as median, 25th and 75th percentiles within the box, with bars showing the minimum to maximum.

3.2.5 The relationship between methylation and gene expression of *BCAT1* and *IKZF1* in colorectal tumour and para-tumour tissues.

The effects of methylation on expression levels of *BCAT1* and *IKZF1* in colorectal tumour and para-tumour tissues were assessed. When comparing the levels of methylation and gene expression of *BCAT1* and *IKZF1* in colorectal tumours tissues and their matching para-tumour tissues, a significant correlation between levels of *IKZF1* methylation and gene expression was observed (R = -0.482; P = 0.002; Figure 19 A). No significant correlation was seen between levels of *BCAT1*

methylation and gene expression in the same cohort of samples (R = -0.017; P = 0.885; Figure 19 B).

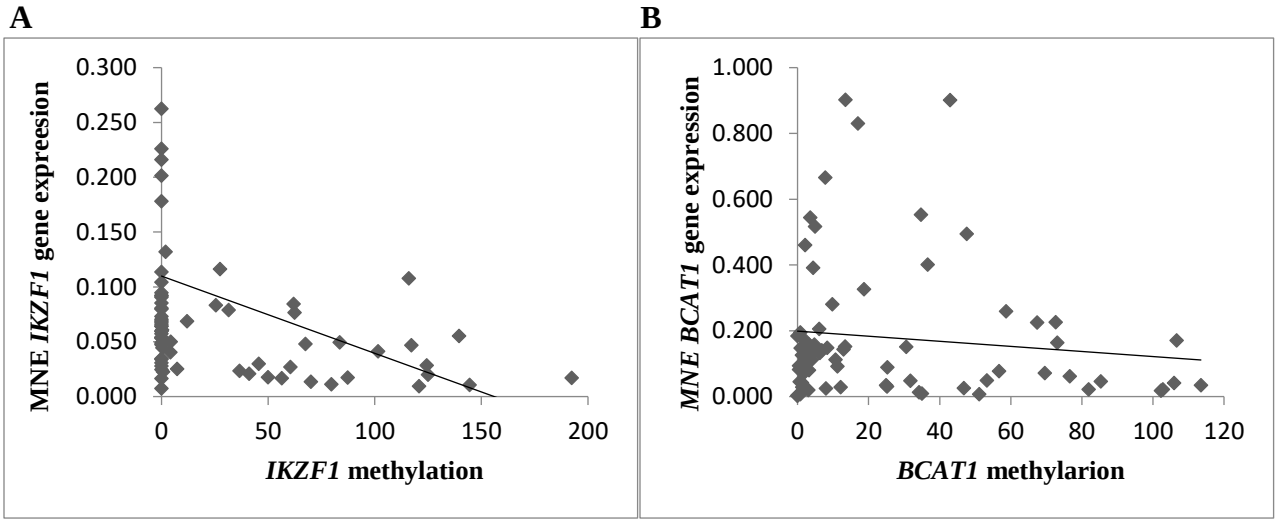

Figure 19. Correlation between methylation and gene expression for colorectal tumour and para-tumour tissues. (A) levels of *IKZF1* methylation and gene expression in colorectal tumours and the matching para-tumour tissues (n = 72) are plotted on x-axis and y-axis respectively, P = 0.002. (B) levels of *BCAT1* methylation and gene expression in colorectal tumours and the matching para-tumour tissues (n = 70) are plotted on x-axis and y-axis respectively, P = 0.885.

Chapter 4. Discussion

CRC is a common disease in the western society. Hypermethylation plays an important role in development of this disease through tumour suppressor gene silencing. Large percentages of CRC cases are subjected to neoadjuvant therapy prior to tumour surgical resection to increase the likelihood of surgical success. However, there are limited non-invasive ways to assess a patient's response to neoadjuvant treatment. This study has shown that colorectal tumours, but not the adjacent non-tumour (para-tumour) tissue, are highly methylated for *BCAT1* and *IKZF1*. Methylation significantly reduced gene expression levels of *IKZF1*, but not *BCAT1*. Therapy reduced methylated *BCAT1* and *IKZF1* levels of the tumour and increased expression of *BCAT1* and *IKZF1*. Levels of methylation may therefore be able to be used as a marker for treatment success prior to surgical intervention.

4.1 Hypermethylation of *BCAT1* and *IKZF1* in colorectal tumour tissue

The role of hypermethylation in human tumorigenesis has been studied intensively (Ehrlich 2002), with DNA hypermethylation in the CpG islands rich promoter regions being found to be associated with cancer development and progression (Esteller 2008). In CRC, an increasing number of genes have been identified as being silenced during tumour development due to hypermethylation events including *APC, MGMT, MLH1, DAPK1, CDH1, CDH13, p14, COX2, RARB2* and *RASSF1A* (Coppede 2014; Kulis & Esteller 2010). These frequently hypermethylated genes are involved in crucial cellular processes, and they mainly function as tumour suppressor genes in normal tissues (Kulis & Esteller 2010).

This current study is the first to evaluate the methylation level of *BCAT1* and *IKZF1* in tumours and matched para-tumour tissues of CRC patients. The qPCR-based methylation assessment

method used in this study was originally developed and optimized for detection of methylation in the gene loci *BCAT1* and *IKZF1* in plasma samples (Pedersen et al. 2015a). When our results were considered as either actual percentages of methylation or as proportions of samples with significant methylation levels, a higher level of methylated *BCAT1* and *IKZF1* in colorectal tumour tissues in relation to para-tumour tissues was observed.

These findings support what has previously been observed in two separate studies that have assessed the levels of methylated *BCAT1* and *IKZF1* in the plasma samples of patients with colorectal tumours (Mitchell et al. 2014a; Pedersen et al. 2015b). These studies used different methods of assessment (genome-wide methylation assessment and quantitative methylation-specific PCR), but both found that *BCAT1* and *IKZF1* were more frequently methylated in the plasma samples from CRC patients than the samples of healthy controls. The relatively high levels of methylated *BCAT1* and *IKZF1* in plasma of CRC patients supports the concept of using these as biomarkers for detection of the presence of colorectal tumours (Pedersen et al. 2015c). As the current study found that tumour tissue, but not the para-tumour tissue, also contained methylated *BCAT1* and *IKZF1*, this indicates that its presence in the blood come from the tumour, rather than just being a general physiological response of the body to the presence of cancer.

In other cancer types, hypermethylation of *IZKF1* has been reported previously. In a study that used next-generation sequencing technology to identify and validate DNA methylated biomarkers for discriminating pancreatic cancer cases from normal controls, *IKZF1* was among the promising candidates that show a higher level of methylation in pancreatic cancer than normal control cases (Matsubayashi et al. 2006). Moreover, Zhang et al. (2013) who found a significant positive correlation between ectopic activation of *IKZF1* and the clinical outcomes of lung cancer patients, reported that DNA hypermethylation predominantly targets the *IKZF1* gene in a cancer-specific

manner. In the context of pituitary tumours, it was found that hypermethylation of exon-1 CpG island, downstream the promoter region of *IKZF1*, was associated with loss of *IKZF1* function and tumour development (Zhu, Asa & Ezzat 2007). This shows that hypermethylation of *IKZF1* is not specific to CRC. In the current study, we also noted that there was no change in levels of methylated *IKZF1* across stages I-IV CRC suggesting that hypermethylation of *IKZF1* in CRC is an early event required for disease development. This is supported by the findings of Zhang et al. (2013) who showed that the hypermethylation of *IKZF1* induced by lung cancer development does not depend on the development stages of the disease.

Similarly, hypermethylation of *BCAT1* has been observed with other cancers, with studies indicating a role of *BCAT1* hypermethylation in lung and glioma tumorigenesis (Diaz-Lagares et al. 2016; Tonjes et al. 2013). *BCAT1* has been proposed as a promising candidate for early diagnosis of lung cancer due to its high frequency of methylation in lung tumour tissues compared with normal controls (Diaz-Lagares et al. 2016). In glioma tumours, the MassARRAY analysis was used to identify the methylation patterns of *BCAT1* promoters. It was shown that both promoters (1 and 2) from which the three *BCAT1* transcripts isoforms (T1, T4 and T6) are generated, undergo hypermethylation in IDH^{mut} (glioma carrying mutant IDH gene) and IDH^{wt} (glioma carrying wild-type IDH gene) respectively (Tonjes et al. 2013). Contrary to DNA hypermethylation, a significant DNA hypomethylation of *BCAT1* was observed in low-malignant potential and high-grade serous epithelial ovarian tumours (Keita et al. 2013). Taken together, these results suggest that cancer induced epigenetic changes in *BCAT1* could be represented by either hypomethylation or hypermethylation events. It is likely that the epigenetic abnormalities of *BCAT1* occur in cancer-specific manners, where in ovarian cancer no changes are observed in *BCAT1* methylation status except for the late advanced stages of the disease indicating that ovarian

cancer-induced *BCAT1* implication is more likely to be a late developmental event restricted to the advanced and metastatic forms of the disease (Keita et al. 2013). On the other hand, the hypermethylation of *BCAT1* that we found in CRC is more likely to be an early event required for cancer growth and development. This could explain why no significant changes in the levels of methylation *BCAT1* across stages I-IV CRC were observed in this study. In agreement with this, *BCAT1* methylation has also been proposed as early event during nasopharyngeal carcinoma pathogenesis (Zhou et al. 2013).

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 the current study could further support the suggestion that the cancer-induced epigenetic abnormalities of both *BCAT1* and *IKZF1* are an early events required for disease development. Therefore, by the time the tumour is clinically present, methylation of *BCAT1* and *IKZF1* has already occurred, and no changes in these levels would be expected as the tumour becomes more aggressive. However, this result could also be because cancer biopsies were taken only from the top of the tumour (the luminal surface of the tumour). This could be investigated further by assessing multiple samples from different areas of the tumours as well as investigating methylation levels in pre-cancerous adenomas.

In the context of levels of methylated *BCAT1* and *IKZF1* in colorectal tumour samples, we have found a positive correlation between the levels. To our best knowledge, there is only one report that has assessed the correlation between levels of methylated *BCAT1* and *IKZF1* during cancer development, but this was not in tissue samples. In agreement with our finding, Pedersen et al. (2015a) reported a significant positive correlation between levels of methylated *BCAT1* and *IKZF1*

in plasma samples of patients with CRC. This suggests that the epigenetic changes are happening to both genes at a similar level and rate.

In this study, there was a wide range in the levels of methylated *BCAT1* and *IKZF1* observed in the non-treated colorectal tumours (values of methylation levels ranged between 0% - 113.5% and 0% -192.5% for *BCAT1* and *IKZF1* respectively). These results may be explained by a number of reasons. First, the inter- and intra-heterogeneity of colorectal tumours. Recent studies have made it clear that there are no two identical tumours (Linnekamp et al. 2015) due to the presence of independent evolution events occurring within the tumour which give rise to a number of subclonal populations of cells defined by distant genetic and epigenetics diversities (Barranha et al. 2015). In fact, it is a combined effect of internal and external forces during tumour evolution, selecting tumoural sub-clones that adapt best to the surrounding environment and drive tumour heterogeneity (Yates & Campbell 2012). Evidence of intra-heterogeneity on DNA methylation patterns in colorectal tumours was evaluated by analysing three different regions of tumour: the region nearest the digestive tract surface, the central bulk, and the invasive front (Martinez-Cardus et al. 2016). The results indicated that colorectal tumour has heterogeneity in DNA methylation patterns in its sub-clonal populations of cells, with the greatest methylation found in the central bulk of the tumour (Martinez-Cardus et al. 2016).

The range in methylation results in the current study may also be due to the architectural structure and tissue composition of the tumours because in addition to neoplastic and normal epithelial cells, tumours contain different stromal infra-structures (Bian et al. 2007; Bosman et al. 2003; Domazet et al. 2008). Stromal contents that are found in colorectal tumours include tumour-associated macrophages, myeloid derived suppressor cells, mast cells, cancer-associated fibroblasts, monocytes, neutrophils, natural killer cells, endothelial cells, endothelial progenitor cells, platelets,

and mesenchymal stem cells (Peddareddigari, Wang & DuBois 2010). Levels of methylated *BCAT1* and *IKZF1* may vary among neoplastic and stromal cells of colorectal tumours. In agreement with this suggestion, a recent report that assessed the levels of methylated *SEPT9* in homogeneous populations of neoplastic, epithelial and stromal cells of colorectal tumours (obtained using laser capture microdissection method) found that levels of methylated *SEPT9* vary among neoplastic and stromal cells of same tumour (Wasserkort et al. 2013).

Both previous points (tumoural polyclonality and tissue constitution and composition) may be affected by the sampling process (Barranha et al. 2015). It is not clear whether biopsy samples are representative of all genetic and epigenetic features of the tumour. One study suggested that a single biopsy sample is sufficiently illustrative of the entire tumour (Fadhil et al. 2012), however at molecular level it has been demonstrated that using one tumour biopsy will misdiagnose about 7% of patients with *K-ras* wild-type mutation in CRC (Richman et al. 2011), and a biopsy from the centre of tumour has different rate of *K-ras* mutation compared with a biopsy from its invasion front (Fadhil et al. 2012). In contrast, tumour biopsies used in the current study were taken only from the luminal surface of the tumours. This may explain the range of methylation values observed in current study. In order to achieve the highest concordance between levels of methylated *BCAT1* and *IKZF1* and resection biopsy, an adequate number of biopsy specimens from different regions of the tumour should be considered. However, taking multiple samples from the tumour was not done in this study, and may not be suitable for future studies because it could interfere with the pathological assessment of tumour for diagnostic purposes.

Apart from intra- and intervariability in epigenetic changes, previously DNA methylation profiles have been found to vary between the colon and the rectal cancers (Kucherlapati 2012), as well as being associated with clinical parameters including gender (Zhang et al. 2011) and age (Jung &

Pfeifer 2015). In this study, we observed that patient demographics including age gender and tumour site had no effect on the levels of methylated *BCAT1* and *IKZF1* in tumour and non-tumour tissues. In agreement with our findings, a study in colorectal tumour tissue found no correlation between age, gender and tumour location and levels of methylated *IKZF1* (Javierre et al. 2011). To our best knowledge, the correlation between *BCAT1* methylation and clinical parameters has not been examined before. We can, therefore, conclude that changes in the level of methylated *BCAT1* and *IKZF1* reported in this study are more likely to be caused by cancer development instead of through the effects of age, gender and/or tumour site.

4.1.1 The effect of cancer-related hypermethylation on *IKZF1* expression.

IKZF1 is a zinc finger transcription factor encoding gene, that regulates gene expression of several target genes through chromatin remodeling. *IKZF1* plays essential roles in regulating immune system function and hematopoietic differentiation (Payne & Dovat, 2011). Expression of *IKZF1* usually leads to the inhibition of transcription of a number of target genes by trans-locating them to the pericentromeric heterochromatin (PC-HC) which is a transcriptionally inaccessible region (Cobb et al. 2000).

A growing body of evidence supports the role of epigenetic changes (hypermethylation) in the abnormalities of *IKZF1* gene expression during carcinogenesis. In the current study a significant downregulation of *IKZF1* gene expression was observed in the colorectal tumour tissues, along with hypermethylation within the *IKZF1* promoter sequence. The downregulation of expression could be a consequence of the hypermethylation, supporting the role of hypermethylation induced abnormalities of *IKZF1* gene expression in CRC. In agreement with our results some cell line studies have reported hypermethylation-induced silencing of *IZKF1* in different cancer types

including lung cancer (Zhang et al. 2013), acute lymphoblastic leukemia (Fang et al. 2009), pituitary gland (Zhu, Asa & Ezzat 2007) and CRC (Javierre et al. 2011).

Whether the functional implications of *IKZF1* alterations participate in CRC progression or not, is not fully determined. However, the observations reported in this study, along with previous observations in other cancer types, suggest that *IKZF1* could function as tumour suppressor gene and its dysfunction due to hypermethylation induced silencing is a potential contributor in cancer growth and development. This suggestion is strongly supported by the findings of Javierre et al. (2011) who reported that the epigenetic deregulation of *IKZF1* is required for the growth of cancer cells line (HCT-116), where the ectopic expression of *IKZF1* in HCT-116 caused a significant reduction in colony formation and cell viability.

Taking into consideration that *IKZF1* represents a complex transcription factor system regulating the expression of a number of target genes including *PTPN6*, *MEIS2*, *GPX7* and *NHLH1* (Javierre et al. 2011) which also have been shown to be affected during carcinogenesis (Peng et al. 2009; Wu et al. 2003), it is possible that deregulation of one or more of *IKZF1* target genes could participate in progression of CRC. Moreover, it could the interaction between *IKZF1* and the other members of Ikaros family of transcription factors-encoding genes (Helios, Aiolos, Eos, and Pegasus) that add additional regulation during CRC progression (Javierre et al. 2011). Therefore, studies are required to identify the possible roles of *IKZF1* target genes and the relationship between members of Ikaros family during colorectal carcinogenesis.

4.1.2 The effect of cancer-related hypermethylation on *BCAT1* expression.

BCAT1 is an oncogene encoding for branched-chain amino acid transaminase enzyme that catalyses the reversible transamination of branched-chain alpha-keto acids to branched-chain L-amino acids. The catabolism products of *BCAT1* are essential for cell growth and they have been

associated with several cellular activities including cell proliferation, cell cycle progression, differentiation and apoptosis (Eden & Benvenisty 1999; Eden, Simchen & Benvenisty 1996; Schuldiner et al. 1996).

The functional role of *BCAT1* in CRC development and progression remains to be elucidated. A recent study suggested that *BCAT1* plays an essential role in metastasis of CRC, and the same study even considered *BCAT1* as a prognostic biomarker for predicting distant metastasis in CRC patients (Yoshikawa et al. 2016). This study also speculated that downregulation of *BCAT1* expression may exert impacts on the synthesis of macromolecules which are essential for controlling cell division, probably at the G1-to-S cell cycle control point (Yoshikawa et al. 2016). In other cancer types, previous studies implicate a functional role of *BCAT1* in carcinogenesis. Overexpression of *BCAT1* stimulated by the upregulation of *c-Myc*, an oncogene and transcription factor that can bind to the *BCAT1* promoter (Ben-Yosef et al. 1998), has been found to be an important early event during nasopharyngeal carcinoma occurrence required for cell growth and progression (Zhou et al. 2013). In epithelial ovarian cancer, the Agilent CpG island microarrays and the ingenuity pathway analysis showed that *BCAT1* dysfunction induced by epigenetic changes is probably related to disease progression, including tumour invasion/metastasis (Keita et al. 2013). This suggestion was further supported by Wang et al. (2015) who reported a significant reduction in ovarian cancer cell proliferation, migration and invasion following knockdown of *BCAT1* gene expression. Further functional roles of *BCAT1* in ovarian cancer development could be exerted through the dysregulation of miRNAs (Zhang et al. 2015), where it has been shown that the downregulation of miRNAs (let-7 or mir-155) which have potential tumor suppressor gene activity, promote ovarian cancer cell growth through the overexpression of *BCAT1*.

Regulation of *BCAT1* expression through epigenetic changes has been reported in few cancer types. We hypothesised that cancer-induced hypermethylation would cause downregulation of *BCAT1* expression in CRC, but in other cancers hypermethylation was shown to cause upregulation of *BCAT1* expression. In the current study *BCAT1* gene expression was shown to be methylation-independent as no significant downregulation in *BCAT1* gene expression was observed. In contrast, Tonjes et al. (2013) reported a correlation between the overexpression of *BCAT1* and its hypermethylation status in glioma tumours while an association between *BCAT1* silencing and hypermethylation status of its promoter was observed in lung cancer (Diaz-Lagares et al. 2016). The contrasting findings of some studies reporting overexpression and others reporting downregulation of *BCAT1* expression in tumour tissues, as well as varying reports on the effects of epigenetic changes on *BCAT1* expression in our study and others could be explained by the tumoural polyclonality and tissue constitution and composition which may be affected by the sampling process as mentioned before. Alternatively, the lack of hypermethylation induced gene expression changes of *BCAT1* in the current study could be explained by the finding that the expression of *BCAT1* transcripts isoforms (T1, T4 and T6) are controlled by two different promoter regions (T1 expression is regulated by promoter 1 while T4 and T6 expression are regulated by promoter 2). In glioma tissue, hypermethylation of promoter 1 was found to cause upregulation of *BCAT1* expression (T1 isoform), while hypermethylation of promoter 2 was associated with downregulation of *BCAT1* expression (T4 and T6 isoforms) (Tonjes et al. 2013). In the current study, the *BCAT1* methylation assay evaluated the methylation status of CpG island 42 located in promoter 1 only while the gene expression assay was designed to assess all the isoforms of *BCAT1* together. Therefore, this probably led to the underestimation of cancer induced gene expression changes of *BCAT1* reported in the current study. While our study has looked at the overall *BCAT1*

expression (all isoforms), future studies could assess the effects of hypermethylation events on the expression of each one of *BCAT1* isoforms separately.

It is possible that *BCAT1* dysfunction could participate indirectly in CRC tumorigenesis through deregulation of several genes controlling the metabolism of CRC cells. This idea is supported by previous observations made in ovarian cancer cells that showed the multiple inhibitory effects that *BCAT1* suppression exerts on numerous metabolism-related genes responsible for catalyzing lipid production and protein synthesis (Wang et al. 2015).

4.2 Methylation and expression of *BCAT1* and *IKZF1* in para-tumour tissue

The genetic and epigenetic modifications observed in colorectal tumours have been previously reported to extend into the tumour surrounding and macroscopically normal mucosa supporting the field cancerization theory (Patel et al. 2015). Field cancerization has been found to occur in different types of malignancies, including lung, colon, prostate and pancreas (Braakhuis et al. 2003). Several studies have discussed the role of field aberrant DNA methylation effects in CRC as pro-tumorigenic mutations that are insufficient to create morphological alternations in the colonic epithelial tissue but are able to pre-dispose to neoplasm development (Luo, Yu & Grady 2014). In agreement, Kim et al. (2006) suggested the early appearance of aberrant DNA methylation during the adenoma-carcinoma sequence could affect genes resulting in CRC initiation and progression. In the concept of field cancerization, there are a number of genes including *MGMT*, *P14^{ARF}*, *EVL*, *APC*, *DKK1*, *WIF1*, *SFRP1*, *SFRP2*, *RUNX3*, *SOCS1*, *NEUROG1* and *CACNA1G* which have been detected to have aberrant methylation status in the morphologically normal colonic mucosae adjacent to colorectal tumours (Luo, Yu & Grady 2014). Moreover, the aberrant methylation status of *MGMT* (Shen et al. 2005) and *EVL* (Grady et al.

2008) was reported to be associated with the distance where the normal-appearance tissues were collected from, with high levels of methylation in tissues localised close to the tumour. This suggests the involvement of these aberrantly methylated genes in the early carcinogenesis events that are more likely to precede and predispose to colorectal malignant transformation later on (Patel et al. 2015).

In the current study, the field cancerization effects of methylated *BCAT1* and *IKZF1* were assessed in para-tumour tissues surrounding colorectal tumours. Interestingly, no field cancerization effects of methylated *BCAT1* and *IKZF1* were observed in para-tumour tissues collected either close to the tumour (distance ≤ 50 mm of tumour) or distant to the tumour (distance > 50 mm of tumour). No correlation was observed between the distance where para-tumour tissues were collected with the levels of methylated *BCAT1* and *IKZF1*. This suggests that changes in levels of methylated *BCAT1* and *IKZF1* could be localized to the tumour itself and the para-tumour tissue is somehow protected from these changes. Another suggestion could be that the changes in methylation of *BCAT1* and *IKZF1* may not occur as an early event in colorectal carcinogenesis, instead it could be related to later stages of CRC progression. This suggestion could be supported by previous observations that describe DNA hypermethylation as late tumourigenesis events (Xie et al. 2015). For examples, in sporadic CRC, the inactivation of *MLH1* gene due to its promoter hypermethylation was found to be related to the transformation of intermediate adenoma to late adenoma that occurs late during the adenoma-carcinoma pathway (Walther et al. 2009). The aberrant methylation of *IKZF1* as a late tumourigenesis event could further be supported by (Javierre et al. 2011) who observed an increase in the levels of methylated *IKZF1* as CRC stages progress, while to our knowledge no stage related methylation changes has been reported in *BCAT1* before. However, in the current study statistical analysis showed no overall changes in the

levels of methylated *BCAT1* and *IKZF1* as the tumour stage progressed. The inconsistency with our results could be due to the study design, where usually the assessment of field cancerization effects is performed by evaluating methylation status of specific loci within the normal-appearance tissue and observing the presence of associations between levels of methylation within the promoter sequences of selective candidate genes and the existence of concurrent neoplastic lesion located somewhere surrounding the primary tumour (Belshaw et al. 2010; Hiraoka et al. 2010; Shen et al. 2005). In contrast, our study was designed to assess the methylation status of selected CpG islands within the promoters of *BCAT1* and *IKZF1* genes. Moreover, with the PCR assay used, *BCAT1* was considered to be methylated only when all cytosines were methylated within the CpG island. Partial methylation would not be detected with the design of the assay. Therefore, the partial methylation status of promoter regions that could be observed by other authors as field cancerization events would not be detected. It is therefore possible that the tissue surrounding the colorectal tumour has some methylation. Similarly, para-tumour tissues could have a partially methylated *IKZF1*. However, the *IKZF1* assay did pick up partial methylation and therefore field effects was not observed. In conclusion, based on the current study, it is more likely that these methylation changes to *BCAT1* and *IKZF1* are localised to the tumour.

4.3 Neoadjuvant therapy

The administration of ionizing radiation to CRC patients as a therapeutic agent prior to tumour surgical resection is known as neoadjuvant therapy (Julien & Thorson 2010). The neoadjuvant therapy is an effective and valuable treatment option in cancer therapy. The main objective for neoadjuvant therapy is to shrink tumour mass making the tumour resection operation more likely to succeed through improving the ability to discriminate between the tumour and its adjacent para-tumour tissues (Glynne-Jones & Chau 2013). The rationale of using ionizing radiation as

neoadjuvant therapy is based on its ability to promote damage in cancer cells by activating a number of complex cellular pathways (Poortmans 2007). Radiotherapy is a well-characterised genotoxic agent that induces cancer cell death by direct (genetic) and indirect (epigenetic) mechanisms. Moreover, several reports have discussed the mechanisms underlying the impact of radiotherapy on DNA methylation patterns of cancer cells (Goodhead 1994); however, there is little information on radiotherapy induced methylation and expression changes of specific genes in cancer (Bae et al. 2015).

Our study is the first that reports the impact of neoadjuvant therapy (radiotherapy) on the levels of methylated *BCAT1* and *IKZF1* in colorectal tumour tissues compared with non-treated colorectal tumour tissues. The high levels of methylated *BCAT1* and *IKZF1* found in the non-treated colorectal tumour tissues were significantly reduced by neoadjuvant therapy. In agreement with our finding, genome-wide DNA methylation analysis showed that the exposure of CRC cells (HCT116) to radiotherapy induces the DNA demethylation (Bae et al. 2015). Moreover, the study demonstrated seven genes (*ANGPT1*, *APBB2*, *CHGA*, *CTGF*, *IFI6*, *IGLON5*, and *SLC43A2*) that are frequently methylated in CRC cells to undergo radiotherapy induced hypomethylation events (Bae et al. 2015). In another comparative study performed on colorectal tumours samples, promoters of two genes (*MGMT* and *APC1A*) were reported to have less methylation in radiated tumours than non-radiated tumours indicating the radiation-induced hypomethylation effects. However, *p16* and *APC1B* promoters contained higher methylation in radiated tumours compared with non-radiated (Krakowczyk et al. 2010). This suggests that radiation does not always decrease the levels of methylation.

The current study has also reported for the first time the effects of neoadjuvant therapy on levels of *BCAT1* and *IKZF1* gene expression. The reduction in methylation of *IKZF1* in colorectal

tumours was accompanied by an increase in the level of gene expression compared to non-treated levels. These findings suggest that the expression of *IKZF1* in colorectal tumour tissues is methylation dependent. The same observations have been made previously. Javierre et al. (2011) reported an increase in the expression of *IKZF1* in CRC cell lines following the partial demethylation of *IKZF1* promoter by 5-aza-2-deoxycytidine (a demethylation agent). Similarly, in the current study, a significant increase in *BCAT1* gene expression was observed in treated tumours compared with non-treated tumours. In agreement, the methylation dependent gene expression of *BCAT1* was reported previously in ovarian cancer where the overexpression of *BCAT1* was significantly associated with the hypomethylated status of its promoter (Wang et al. 2015).

Moreover, we have examined whether the decrease in methylation and the increase in expression of *BCAT1* and *IKZF1* were due to the effects of the combined neoadjuvant therapy (chemotherapy and radiotherapy) or due to radiotherapy alone. While this is a small sample size, we observed no significant differences in the levels of methylation and gene expression of *BCAT1* and *IKZF1* between the 65% of tumour tissues subjected to chemotherapy and radiotherapy, and the 29% of tumours that received radiotherapy as the only treatment. This suggests that the demethylation and the overexpression of *BCAT1* and *IKZF1* were due to influences of radiotherapy (ionizing radiation) on colorectal tumour tissue. One explanation to the radiotherapy induced global hypomethylation effects (Pogribny et al. 2005), could be that the exposure to radiotherapy caused a downregulation in the expression levels of methyltransferases (DNMT1 and DNMY3a) and methyl CpG binding proteins (Loree et al. 2006; Pogribny et al. 2004; Raiche et al. 2004). Another possible explanation for the neoadjuvant-induced hypomethylation effects is that exposure to radiation may cause re-localization of methyltransferases from the nucleus to the cytoplasm (Bae

et al. 2015; Razin & Riggs 1980). This re-localization, in turn, could result in an alteration in the DNA methylation maintenance machinery in the genome.

Reduction in tumour size as consequences of the exposure to treatment varies among CRC patients (as shown in Table 12). More than one third of the tumours had shrunk more than 50% of their original size. In addition to the reduction in tumour size there was also a downstaging of the tumour after the exposure to treatment in all but four patients. Size reduction and downstaging of tumours after radiotherapy treatment have been reported previously (Horn, Morild & Dahl 1990). In the context of tumour radiosensitivity, it is believed that intrinsic heterogeneity of stem cells within tumour is driving the differences in tumour radiosensitivity, and the presence of the most radioresistance clone (stem cells) determines tumour curability through the ability to reconstitute tumour growth after exposure to radiotherapy (Baumann, Krause & Hill 2008). More recently a study showed that the likelihood of tumour response to radiotherapy could be determined by the presence of several components not only in the tumour itself but also in its surrounding microenvironment (Barker et al. 2015). For example, Garcia-Barros et al. (2003) revealed that abundance of endothelial elements within the tumour plays an important role in regulating tumour radiosensitivity through the microvascular endothelial apoptosis enhancement which is essential for sustained microvascular dysfunction and eventually tumour growth regression.

Of special interest, we observed an association between the treatment-induced reduction of tumour size and levels of methylated *BCAT1* and *IKZF1*. In high responding tumours, levels of methylated *BCAT1* and *IKZF1* were lower than the levels in low responding tumours. This finding suggests that the levels of methylated *BCAT1* and *IKZF1* could be used as a prognostic biomarker in CRC that may predict an improved response to radiotherapy or treatment-responsive tumours in general. Similarly, a correlation between methylation levels and tumour response to radiotherapy was

reported previously in breast cancer (Halvorsen et al. 2014). The study suggested a panel of four genes (*PDCD1*, *BCAN*, *PCKI* and *GYPE*) that showed high levels of methylation in low response tumours compared to high response tumours, as prognostic markers to predict breast tumour response to radiotherapy (Halvorsen et al. 2014). The findings in the current study of different methylation levels with different therapy responses could either be due to the levels of methylation predicting the response to radiotherapy (i.e. tumours with initial low methylation levels having better responses), or due to a direct relationship between the radiotherapy effect and the resulting methylation levels (i.e. if radiotherapy is successful at shrinking the tumour then it also reduces methylation). While no report has linked the methylation status of *BCAT1* and *IKZF1* with predicting and determining the response of CRC patients to neoadjuvant therapy, the differential methylation of some genes has been investigated as predictive markers of neoadjuvant therapy response (Williamson et al. 2015). For example, a significantly greater response to radiotherapy has been observed in mismatch-repair-deficient cell lines, obtained from patients with hereditary non-polyposis CRC, with a hypermethylated *ATM* gene promoter, compared with these cells with hypomethylated *ATM* gene promoter (Kim et al. 2002). While the current study does not have biopsies from each tumour before and after treatment, we have shown that more than 70% of the non-treated tumours have high methylation levels, and therefore it is unlikely that the high response tumours had low methylation prior to treatment.

In the current study, the associations between tumour response to treatment (measured by size shrinkage) and the reductions in levels of methylated *BCAT1* and *IKZF1* were not accompanied by significant changes in expression of the two genes. This finding could be explained by the study design where the tumour's negativity for the methylation assay does not necessarily mean that radiotherapy caused entire demethylation of promoter sequences of *BCAT1* and *IKZF1*, and instead

partial methylation could present (which would not be detected with the assay design). Partial methylation within the promoter sequences of *BCAT1* and *IKZF1* may explain why there were no associations between treatment induced demethylation and gene expression in the high response tumours. Also, it is important to mention that the average time period between the late dose of treatment two which patients were subjected and surgical resection was 68.7 days. This time interval could have allowed tumours to recover their gene expression profile.

4.3.1 The bystander effects of neoadjuvant therapy

Radiation-induced bystander effects were reported early in the 1950s, as the damaged chromosomal structures were noticed in the blood of patients exposed to radiotherapy (Najafi et al. 2014). Bystander effects of radiotherapy could lead to tumourigenesis in distant tissues as consequences of epigenetic mediated genetic alterations (Lomax, Folkes & O'Neill 2013; Mothersill & Seymour 2002). Investigation of the epigenetic regulation of radiation induced-bystander effects showed a global loss of DNA methylation associated with alterations in levels of key proteins that control methylation patterns including DNMT3a, methyltransferase and MeCP2, methyl-binding protein in the bystander skin and spleen tissues seven months following the exposure of the head to localized radiation (Koturbash et al. 2007; Kovalchuk & Baulch 2008). In the current study, there were no obvious changes in level of methylation of *BCAT1* and *IKZF1* in para-tumour tissues treated with radiotherapy (total of 25 Gy or 50 Gy) compared with non-treated para-tumour tissues, suggesting no radiotherapy induced bystander effects on methylation levels of *BCAT1* and *IKZF1*. This suggests that methylated *BCAT1* and *IKZF1* may not be involved in the bystander effects signaling system that either promotes apoptotic responses or genomic instabilities in the bystander para-tumour tissues (Ilnytskyy & Kovalchuk 2011). It could be that the bystander exposure to radiation led to accumulated DNA damage and changes in DNA

methylation (Raiche et al. 2004; Tamminga & Kovalchuk 2011) that could eventually cause the elimination of affected cells from the para-tumour tissues by the apoptotic pathway. Another explanation could be the presence of efficient mechanisms that either maintain the normal methylation level or promote healing activities to retrieve the standard methylation profile within the bystander para-tumour tissues (Krakowczyk et al. 2010).

4.4 Study limitations

One of the limitations in the current study was the small sample size. Discrimination in the levels of methylation and gene expression of *BCAT1* and *IKZF1* between treated and non-treated groups was based on the results of 50 CRC patients out of which only 14 patients were subjected to neoadjuvant treatment. Similarly, the observations made regarding the effect of treatments (radiotherapy with or without chemotherapy) on methylation and gene expression of *BCAT1* and *IKZF1* were based on results of 14 treated cases out of which only 5 cases were exposed to the combined therapy (radiotherapy with chemotherapy). It is preferable to use larger population sizes to perform more reliable statistical analyses. Another limitation is that the study did not collect tumour biopsies from each patient prior to being subjected to treatment. It is preferable to determine the effects of treatment on levels of methylated *BCAT1* and *IKZF1* and gene expression by comparing the results of two biopsies from each patient after and before the treatment. A further technical limitation is that the primers sets and probes in the methylation qPCR assay for detection the levels of methylation *BCAT1* and *IKZF1* were designed to bind to only one CpG island. While *IKZF1* only appears to have one CpG island within the promoter region, *BCAT1* has two alternative promoter regions- promoter one contains CpG island 42 while the second promoter contains CpG island 70 (Tonjes et al. 2013). The current study only assessed methylation of CpG island 42. Methylation results that are based on evaluation of multiple islands within the promoter using

pyrosequencing method may be more quantitative and reproducible (Diaz-Lagares et al. 2016). However, the method chosen in the current study was to match the primer/probe design previously applied to blood samples from CRC patients. This CpG island was found to be frequently methylated in plasma from CRC patients (Mitchell et al. 2014a; Pedersen et al. 2015a; Pedersen et al. 2015c).

4.5 Significance of the study

While previous studies have shown an elevated level of methylated *BCAT1* and/or *IKZF1* in plasma of CRC patients (Pedersen et al. 2015c), the current study has shown that tumour tissue has high levels of methylated *BCAT1* and *IKZF1* which is not found in para-tumour tissues. This supports the theory that the appearance of methylated *BCAT1* and *IKZF1* in the circulation come directly from the tumour, strengthening the use of blood analysis for non-invasive detection of CRC as well as for the detection of residual tumour after resection. An assay for evaluating the presence of these two methylation DNA markers may therefore help reduce the mortality rates from CRC and improve population public health through providing a highly accurate and non-invasive screening method for early detection of CRC. The neoadjuvant-induced reduction in methylation observed in *BCAT1* and *IKZF1* might also be reflected in the levels of methylation of these genes in circulating tumour DNA in plasma of patients with colorectal tumours. This in turn could support the concept of using the levels of plasma DNA methylation biomarkers to determine and monitor responses to neoadjuvant therapy. Timely assessment of response could allow not only the discrimination of neoadjuvant-responsive patients from non-responsive patients, but also to avoid the use of non-effective treatments and the exposure to unnecessary toxicity caused by treatments.

Chapter 5. Appendix

Appendix A. Individual patient results used for methylation analysis. Percentages of methylation levels of *BCAT1* and *IKZF1* of tumour and para-tumour tissues from patients with CRC. Levels of methylation were normalized against the percentages of *ACTB* present.

	Para-tumour tissue		**Tumour tissue**	
ID	**% *BCAT1* methylation**	**% *IKZF1* methylation**	**% *BCAT1* methylation**	**% *IKZF1* methylation**
16	4.7	0	7.8	0
27	0.6	0	25.2	0
28	11.6	0	0	0
235	1.2	0	76.6	192.5
396	18.7	0	47.6	45.6
495	6.8	0	30.6	124.4
501	1.2	0	106	79.7
560	3.7	0.4	0	0
644	3.8	0	67.4	116.1
689	3	0	31.8	0
708	8.8	0	6.1	5.3
757	3.6	0	102.3	67.6
780	11.2	0	81.9	83.6
804	58.7	0	6.1	41.2
822	11.6	0	0	0
861	12.9	7.4	10.7	0
918	1.9	0	13.5	31.5
924	1.9	0	51.1	60.5
926	6.6	1	0	0
965	0	0	0	0
966	4.4	0	69.6	0
982	2.1	0	0	27.5
1010	1.5	0	26.3	14.4
1056	0	0	24.9	4.4
1279	8	0.3	34.7	25.5
1340	0.8	0	0.8	139.6
1411	20.9	0	0	0
1515	25.3	1.9	56.7	50

1623	4.9	0	113.5	101.7
1681	1.3	0	13.4	125.1
1784	2	0	73.1	144.6
1811	0.8	0	1	2.5
1979	8.3	0	17	0
2298	1.8	0	15.8	53.7
2322	1.5	0	53.3	0
3002	0.9	0	106.7	117.2
3017	0.4	0	36.6	0.8
3024	4	0.7	2.9	31.5
3057	2.1	0	35	36.7
3079	3.2	0	0	0
3108	3.1	0	34.2	70.1
3153	11.8	0	117.5	114.4
3185	9.8	0	46.8	56.5
3317	0.4	0	42.9	62.5
3393	4.1	0	85.3	87.3
3414	6.3	0.4	102.8	120.9
3418	50.5	76	1	0.9
3424	1	0	72.6	62
3446	12.1	4.2	7.7	12.1
3474	12.7	0	0	0

Appendix B. Individual patient results used for gene expression analysis. Values of take-off points of *BCAT1*, *IKZF1* and *HPRT1* obtained from Rotor-Gene® Q instrument real-time PCR (Qiagen). The relative gene expression of *BCAT1* and *IKZF1* in tumour and para-tumour tissue samples was normalized against the expression of *HPRT1* and expressed as the Mean Normalized Expression (MNE) using *Q-Gene* application (Simon 2003).

ID	Para-tumour tissue						Tumour tissue					
	***BCAT1* expression**			***IKZF1* expression**			***BCAT1* expression**			***IKZF1* expression**		
	Take-off point of reference gene	**Take-off point of target gene**	**MNE**	**Take-off point of reference gene**	**Take-off point of target gene**	**MNE**	**Take-off point of reference gene**	**Take-off point of target gene**	**MNE**	**Take-off point of reference gene**	**Take-off point of target gene**	**MNE**
16	24.9	29.55	0.158	24.9	29.3	0.049	24.25	26.4	0.665	24.25	27.45	0.104
27	24	30.75	0.045	24	27.4	0.092	24.55	32	0.031	24.55	28.7	0.057
28	24.65	28.3	0.281	24.65	28.5	0.069	28.15	33.25	0.136	28.15	32.65	0.044
235	26	33	0.042	26	30.95	0.034	23.15	29.3	0.062	23.15	29.25	0.017
396	31.4	35.2	0.326	31.4	31.6	0.638	26.6	29.4	0.494	26.6	31.75	0.030
495	24.8	29.65	0.140	24.8	28.65	0.069	22.7	27.3	0.151	22.7	28	0.028
501	25.9	31	0.126	25.9	29.7	0.070	23.2	30.05	0.041	23.2	29.95	0.011
560	25.2	30.45	0.113	25.20	28.15	0.121	24.85	27.8	0.426	24.85	27.5	0.146
644	24.4	29.35	0.131	24.4	26.55	0.201	24.1	28.1	0.225	24.1	27.25	0.108
689	28.6	37	0.020	28.6	28.7	0.700	20.85	27.3	0.048	20.85	26.2	0.028
708	x	x	x	x	x	x	25	27.3	0.626	25	27	0.220
757	28.5	31.25	0.544	28.5	30.4	0.226	26.25	34.75	0.018	26.25	30.65	0.048
780	24.26	29.8	0.092	24.26	26.3	0.216	23.7	31.7	0.022	23.7	28.1	0.049
804	26.45	30.35	0.259	26.45	30.5	0.060	21.55	25.55	0.206	21.55	27.35	0.021
822	24.5	28.45	0.235	24.5	29.22	0.040	27.02	30.25	0.390	27.02	27.9	0.436
861	24.55	29.35	0.143	24.55	30	0.025	21.8	26.85	0.113	21.8	26.8	0.035
918	25.25	30.35	0.123	25.25	29.5	0.053	24.46	26.1	0.903	24.46	28.1	0.079
924	23.5	29.4	0.073	23.5	27.4	0.068	24.25	34.1	0.007	24.25	29.6	0.027
926	25.25	34.05	0.014	25.25	31.45	0.016	26.6	28.4	0.886	26.6	29.3	0.139
965	x	x	x	x	x	x	25.56	25.75	2.185	25.56	28.35	0.133
966	24.1	27.15	0.392	24.1	26.45	0.178	24.5	30.5	0.071	24.5	30	0.025
982	23.46	27.9	0.170	23.46	27.1	0.080	25.95	30.4	0.185	25.95	28.95	0.116

1010	27.3	0	0.000	27.3	29.3	0.215	25	28.5	0.311	25	28.7	0.076
1056	20.35	31.7	0.003	20.35	26.55	0.016	22.5	29.6	0.035	22.5	26.9	0.050
1279	24.6	32.4	0.025	24.6	28.65	0.061	24.8	27.3	0.553	24.8	28.35	0.083
1340	27.9	38	0.007	27.9	31.45	0.081	25	29.3	0.195	25	29.2	0.055
1411	26.3	33	0.050	26.3	30.6	0.051	27.05	31.05	0.249	27.05	29.4	0.173
1515	25.75	31.45	0.088	25.75	28.55	0.132	22.65	28.4	0.077	22.65	28.7	0.018
1623	26.2	28.9	0.517	26.2	27.9	0.262	22.65	29.8	0.034	22.65	27.35	0.041
1681	24.75	32.35	0.028	24.75	29.2	0.047	20.5	24.95	0.153	20.5	26.4	0.020
1784	25	29.9	0.137	25	28.05	0.114	20.05	24.35	0.164	20.05	26.95	0.011
1811	24.75	28.95	0.205	24.75	29	0.054	26.2	28.75	0.564	26.2	30.2	0.062
1979	28.8	33.8	0.148	28.8	29.15	0.597	22.25	23.9	0.831	22.25	26.05	0.073
2298	23.35	30.15	0.043	23.35	28.85	0.025	23.48	25.95	0.538	23.48	27.75	0.054
2322	24.5	29.9	0.101	24.5	28.75	0.054	22.1	28.6	0.049	22.1	29.55	0.007
3002	24.5	29.25	0.147	24.5	27.85	0.095	26.82	31.46	0.170	26.82	31.25	0.047
3017	23.65	29.35	0.082	23.65	27.9	0.054	23.1	26.05	0.401	23.1	28.75	0.023
3024	25.68	32.4	0.049	25.68	30.7	0.033	25.3	31.1	0.082	25.3	29.3	0.062
3057	29.6	32.7	0.461	29.6	33.6	0.060	23.52	32.9	0.010	23.52	29.1	0.024
3079	24.74	30.55	0.080	24.74	28.7	0.064	27.2	38.8	0.003	27.2	32.3	0.031
3108	26.46	31.2	0.159	26.46	30.35	0.066	24.54	33.45	0.013	24.54	31	0.013
3153	24.16	30.02	0.076	24.16	29.22	0.032	24.6	30.16	0.092	24.6	28.6	0.063
3185	25.4	29.1	0.281	25.4	28.9	0.085	22.6	30.25	0.025	22.6	28.75	0.017
3317	25.7	31.3	0.094	25.7	29.1	0.091	23.75	25.35	0.901	23.75	27.45	0.077
3393	22.8	27.9	0.113	22.8	26.8	0.064	24	30.7	0.046	24	30.05	0.017
3414	24.8	29.75	0.133	24.8	29.35	0.044	24.6	32.65	0.022	24.6	31.6	0.010
3418	25.4	35.6	0.006	25.4	32.3	0.010	25.6	31.7	0.070	25.6	29.25	0.078
3424	23.84	29.6	0.080	23.84	27.25	0.092	23.45	27.4	0.227	23.45	27	0.084
3446	24.9	32.5	0.028	24.9	29.6	0.040	28.2	0	0.000	28.2	32	0.069
3474	25.16	29.8	0.161	25.16	29.95	0.038	27.76	34.65	0.047	27.76	30.64	0.123

X represents no amplification of reference gene due to poor quality RNA

Chapter 6. References

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