Molecular Roles of Short Chain Fatty Acids in Colorectal Cancer

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Declaration

I, Dr. Cu-Tai Lu, certify that

i. This thesis comprises only my original works including the design, collection and analysis of data, towards the degree of Masters by Research

ii. That all research procedures reported in the thesis received the approval of the relevant Ethics/Safety Committees

iii. Due acknowledgement has been made in the text to all other material used

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Abstract

Colorectal cancer is the third most common malignancy in the western world in respect to incidence and mortality rates. In Australia, colorectal cancer was the second most common registrable cancer in 2001 with 12,844 new cases reported and since 1991 both male and female incidence rates have increased each year by an average of 0.3% and 0.1% respectively. Despite advances in both surgical resection technique, and adjuvant and neo-adjuvant chemo-radiotherapy, unless the cancer is detected at a very early stage, long term and disease-free survival rates are less than 100%. Prevention is the key to achieving long term survival.

Low fibre diet is considered to be one of many possible causes of colorectal cancer. It has been suggested that high dietary fibre intake reduces the risk of colorectal cancer by increasing stool bulk and reducing transit time, thus minimising exposure of colorectal mucosa to potential carcinogens, while also altering microbial composition and reducing intraluminal pressure. With advances in molecular biology, the focus of research has shifted from the protective effects of a high fibre diet to the study of short chain fatty acids.

Butyrate acid has been implicated as potentially the most significant short chain fatty acid in protecting the colorectal mucosa against colorectal cancer. Butyrate is a product of the bacterial fermentation of undigested dietary fibre in the lumen of the colon and rectum. It is the ligand to the short chain fatty acid receptors GPR43 and GPR41. At the cellular level, butyrate induces apoptosis by inhibition of histone deacetylase activity and induction of $p21^{Waf1/Cip1}$ expression.

The study aims to assess by immunohistochemistry and real-time RT-PCR, the expression of GPR43 and GPR41 in human colorectal mucosa and tumours, and the effects of butyrate on GPR43 and GPR41 in colorectal cancer cell lines. Immunohistochemical staining of paired, same subject normal colorectal mucosa and tumours suggested a significant down regulation of GPR43 and GPR41 in tumours. Butyrate reduced the GPR43 expression across three concentrations in HT-29. In summary, while more research is needed, GPR43 and GPR41 may play a role in mediating the apoptotic effects of butyrate.

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List of Abbreviations

5-FU	5-Fluorouracil
ACPS	Australian Clinico-Pathological Staging
ACTB	Beta Actin
AJCC	American Joint Committee for Cancer
AOAC	Association of Official Analytical Chemists
Apaf-1	Apoptosis-activating factor-1
APC	Adenomatous Polyposis Coli
cAMP	Adenosine 3´,5´-cyclic phosphate (cyclic AMP)
CAPP1	Concerted Action Polyposis Prevention 1
CAPP2	Concerted Action Polyposis Prevention 2
CEA	Carcinoembryonic antigen
Cdk2	Cyclin-dependent kinase 2
CHO-K1	Chinese Hamster Ovary cell
COS-7	COS-7 African Green monkey kidney cell line
CRC	Colorectal cancer
D1-Cdk 4,6	Cyclin D-dependent 4,6
DCC	Deleted in Colorectal Carcinoma
DMH	1,2-dimethylhydrazine
DNP	1,8-dinitropyrene
E-Cdk2	Cyclin E-dependent 2
ERK1/2	p42 and p44 mitogen-activated protein kinases
FAP	Familial Adenomatous Polyposis
FBS	Fetal bovine serum
FLIPR	Fluorometric imaging plate reader
FOLFOX	FOL– Fluorouracil, F – Folinic acid (leucovorin), OX – oxaliplatin
FSANZ	Australia/New Zealand food authorities
$G\alpha_i$, $G\alpha_o$, $G\alpha_q$	Heterotrimeric G proteins, $G\alpha_i$, $G\alpha_o$, $G\alpha_q$
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenase
GPCRs	G protein-coupled receptors
GPRs	G protein-coupled receptors
GPR41	G protein-coupled 41 receptor
GPR43	G protein-coupled 43 receptor
GPRs	G protein-coupled receptors
H3	Histone 3

H4	Histone 4
HAT	Histone acetyltransferases
HDAC	Histone deacetylase
HEK293T	Transfected Human Embryonic Kidney 293 cell
hGPR43	Human G protein-coupled receptor 43
hGPR41	Human G protein-coupled receptor 41
HNPCC	Hereditary Non Polyposis Colon Caner
IGF-1	Insulin-like growth factor-1
IP_3	Inositol 1,4,5-triphosphate
LPS	Lipo-polycharride
LVI	Lymphovascular Invasion
mRNA	Messenger Ribonucleic Acid
MCT1	Monocarboxylate transporter 1
MHC I	Major Histocompatibility Complex I
MMR	Mismatch repair
OR	Odd Ratio
$p21^{wafl/Cip1}$	$p21^{wafl/Cip1}$ protein
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLCβ	Phospholipase C
PTX	Pertussis toxin
Rb	Retinoblastoma protein
RCT	Randomised controlled trial
Real-time RT-PCR	Real-time reverse transcriptase polymerase chain reaction
RPLP0	Ribosomal protein large P0
RR	Relative risk
rRNAs	Ribosomal RNA
SCFA	Short chain fatty acid
SD	Standard deviation
TAE	Tris, Acetic acid, EDTA
TBS	Tris-buffered Saline
TBSB	Tris-buffered saline bovine serum albumin
THP-1	Human monocyte THP-1 cell line
TME	Total mesorectal excision
TNF	Tumour necrosis factor
TNM	Tumour depth, Nodal status and Metastatic status

TRAIL	TNF-Related apoptosis-inducing ligand
TSA	Trichostatin
UICC	Union Internationale Centre le Cancer
β-ME	β-Mercaptoethanol

Chapter One: Introduction

Overview

CRC is a common cancer. Dietary fibre is known to have the protective effects against CRC. The recent discovery of short chain fatty acids receptors, namely GPR43 and GPR41, re-ignited intense research into the protective effects of dietary fibre. This chapter reviews CRC, the evidence behind the protective effects of dietary fibre, short chain fatty acids receptors and butyrate including the aims and hypotheses.

1.1 Colorectal cancer

Colorectal cancer (CRC) is a malignant tumour that arises from the bowel wall, one of the most common cancers in Australia (Figure 1.1). It is second only to lung cancer as a cause of cancer related death. In the western world, it is the third most common malignancy in respect to incidence and mortality rates (Jemal et al. 2005). It is estimated that 5% of the population, or 1 in 21 people, will develop colorectal cancer (Calvert & Frucht 2002). In Australia, colorectal cancer was the most common registrable cancer in 2001 with 12,844 new cases reported. In males it was the second most common registrable cancers after prostate cancer, (6,961 new cases diagnosed in 2001) and in females it was the second most common registrable cancer after breast cancer (5,883 new cases diagnosed in 2001). It was third in order of cancers causing death, being responsible for 13% of Australian cancer deaths in 2001 (Australian Institute of Health and Welfare 2001).

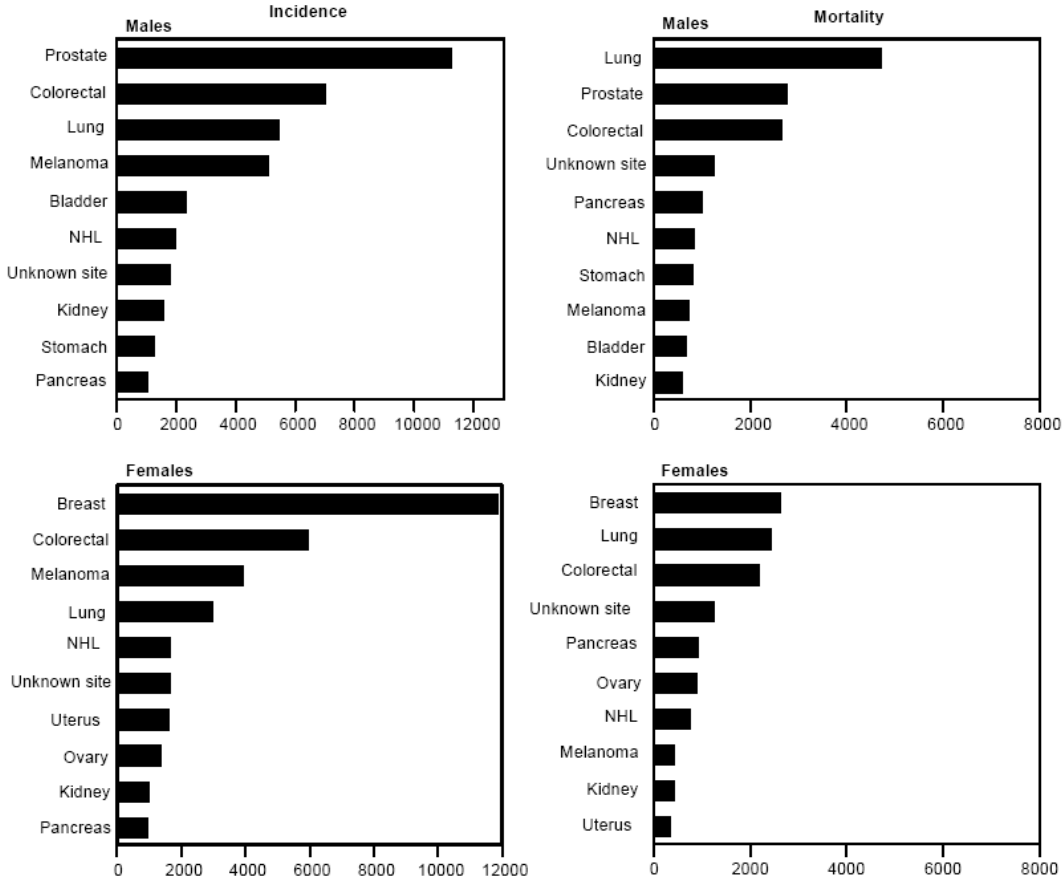


Figure 1.1: Cancer incidence and mortality in 2001, Australia (Adapted Australian Institute of Health and Welfare, 2001)

There are about 12,600 new cases of colorectal cancer diagnosed and accounted for about 4700 deaths annually. The incidence rate of colorectal cancer is increasing, with male and female incidence rates having increased by an average of 0.3% and 0.1% respectively per year since 1991. Mortality rates, however, have fallen steadily, decreasing by 1.2% and 1.6% for males and females respectively per annum between 1991 and 2001 (Australian Institute of Health and Welfare 2001).

The individual lifetime risk of developing colorectal cancer is one in 21, with greater risk after the age of 40 which then progressively increases from the age of 50. Males face a higher risk than females, the lifetime risk before the age of 75 being 1 in 17 and 1 in 26 for males and females respectively (Australian Institute of Health and Welfare 2001).

1.1.1 Clinical Features

Early non-specific symptoms of colorectal cancer include per rectum bleeding, loss of appetite, altered bowels habit and symptoms of iron deficiency anaemia. Late non-specific symptoms include loss of weight, abdominal pain and mass, overt per rectum bleeding and tenesmus in low rectal cancer. Right-sided cancer normally presents with symptoms of anaemia. Up to 20% of patients have stage IV disease at the time of presentation. Colorectal cancer is diagnosed by colonoscopy and histopathological examination of the biopsies (Mella et al. 1997).

1.1.2 Diagnosis

Diagnosis of CRC is based on a complete history, physical examination including digital rectal examination and colonoscopic examination of the colon. Colonoscopic examination is considered to be the diagnostic of choice as it allows direct visual observation of the tumour and its site in the colon. Tissue diagnosis is possible with the biopsy channel of the colonoscope.

1.1.3 Aetiology

The aetiology of colorectal cancer is believed to be multi-factorial. Cancer develops over multiple changes in cell DNA, by either the acquiring of oncogenes, the loss of tumour suppressor genes or the loss of mismatch repair genes. The pathogenesis is complex and involves interaction of both genetic and environmental factors (Fearon 1994; Potter, J. D. 1995; Reddy, B et al. 1989).

The majority of the colorectal cancers are sporadic in origin and the result of somatic mutations. Less than 30% of patients have a family history of colorectal cancer (Prichard & Tjandra 1998; Tjandra 2001). Of these hereditary colorectal cancers, only 5% belong to well defined familial genetic syndromes such as Hereditary Non Polyposis Colon Caner (HNPCC) and Familial Adenomatous Polyposis (FAP) (Hardy, Meltzer & Jankowski 2000).

1.1.4 Risk factors

Risk factors can be divided into genetic and environmental. Of the genetic factors, a family history of either colorectal cancer/adenoma or gynaecological cancer is by far the most

significant. A known genetic mutation among family members is crucial but unknown mutation is just as significant. Personal history of CRC is the second most important risk factor. Being aged 50 or over and having a history of inflammatory bowel disease are also well known risk factors (Burt & Peterson 1996). Environmental factors including low dietary fibre intake, high levels of red and processed meat and fat consumption, low levels of physical activity, excess alcohol intake, and tobacco smoking increase the risk of developing CRC (COMA 1998; Fund 1997; Potter, John D. 1996).

1.1.5 Protective factors

The risk of developing colorectal cancer can be reduced. High dietary fibre intake in the form of fresh fruit, vegetables and cereal can protect against colorectal cancer (Bingham et al. 2003; Freudenheim et al. 1990; Fuchs et al. 1999; Howe et al. 1992; Mathew et al. 2004; McIntyre, A, Gibson & Young 1993; Slattery et al. 2004). Natural occurring dietary micronutrients such as calcium, folate, antioxidants and phytonutrients found in fruits, vegetables and cereals can also reduce the risk. Chemoprevention agent such as aspirin has been shown to minimise the risk of colorectal cancer (Chan et al. 2004; Friis et al. 2003; Thun, Namboodiri & Heath 1991).

1.1.6 Pathophysiology

Colorectal cancer is a carcinoma arising from the colonic epithelium or rectal mucosal layer before spreading through the bowel wall. The mode of metastasis is via the lymphatic system to the regional lymphatic nodes and/or through the haematogenous system to other solid organs such as the liver and lungs.

It is believed that the majority of colorectal cancers arise from adenomas. The “adenoma-carcinoma sequence” is denoted where there is a time lag of between five and ten years (Fearon 1994; Tjandra 2001). Recent reports, however, suggest a relatively new type of serrated polyp with greater malignancy potential and faster rate of progress to carcinoma (Jass, Young & Leggett 2002). Based on autopsy studies and lifetime cumulative incidence of colorectal cancer, it is thought that only about 5% of the adenomas undergo malignant changes. Flat adenomas are considered to be more aggressive and to cause highly malignant adenocarcinomas (Minamoto et al. 1994; Muto et al. 1985; Tsuda et al. 2002). Adenomas are categorised into three histological types: tubular, tubulo-villous and villous adenomas. Malignancy potential is categorised as low and high grade dysplasia. Large, high grade

dysplastic villous architecture adenomas are more likely to harbour foci of carcinoma (Kuramoto et al. 1990). Colorectal cancers known as “de novo” cancer can also arise from flat mucosa (Bedenne et al. 1992; Kanazawa, Watanabe & Nagawa 2003; Watanabe & Muto 2000).

1.1.7 Adenoma-Carcinoma sequence

Fearon and Vogelstein (1990) described the development of sporadic colorectal cancer as a multi-step model of carcinogenesis (Figure 1.2). Accumulated genetic mutations advance the uninhibited growth, proliferation, reduced apoptosis and final carcinoma transformation of the affected cells. It is suggested that at least four or five genetic mutations are necessary for the development of malignancy. This model suggests that activation of oncogenes, loss of tumour suppressor genes and the accumulation of multiple genetic mutations, rather than the sequence of mutations, determines the malignancy potential of the tumour.

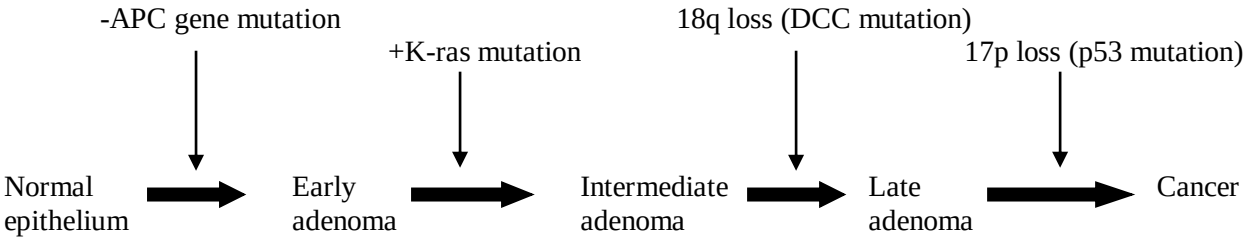


Figure 1.2: Adenoma-carcinoma sequence (Adapted Fearon and Vogelstein ,1990)

Tumour suppressor genes are APC (adenomatous polyposis coli) located on chromosome 5q, DCC (Deleted in Colorectal Carcinoma) gene identified on chromosome 18q, and p53 located on chromosome 17p. K-ras is an oncogene on chromosome 12, it encodes a protein that acts as a one-way switch for the transmission of extracellular growth signals to the nucleus and regulates cellular signal transduction. In FAP the germ line mutation is always the APC gene whereas in HNPCC one of the MMR (Mismatch Repair) genes is lost leading to a defective DNA repair mechanism in DNA replication.

1.1.8 Staging of the colorectal cancer and prognosis

Staging of colorectal cancer is dependent on the imaging technique used (eg: Computed Tomography) and the histopathological examination of tumour samples for depth of invasion beyond the mucosa and the status of lymph node involvement. There are multiple staging

systems. The pathological TNM (Tumour depth, Nodal status and Metastatic status) is a widely used international staging system. Use by institutions of other systems such as the Dukes' staging system and the ACPS (Australian Clinico-Pathological Staging) system reflect familiarity with protocols and procedures (Davis & Newland 1982; Dukes 1932).

<table>
<tr><th>pTNM system</th><th>Histopathology staging</th><th>Dukes' Stage</th><th>ACPS Stage</th><th>Stage</th><th>Prognosis 5 year-survival rate for CRC</th><th>Prognosis 10 year-survival rate for CRC</th></tr>
<tr><td colspan="7">Tumour status</td></tr>
<tr><td>Tis</td><td>Carcinoma in situ</td><td></td><td>A0</td><td></td><td>100</td><td>100</td></tr>
<tr><td>T1</td><td>Submucosa</td><td>A</td><td>A</td><td rowspan="2">I</td><td rowspan="2">75-90</td><td rowspan="2">60-80</td></tr>
<tr><td>T2</td><td>Muscularis propria</td><td rowspan="3">B</td><td rowspan="3">B</td></tr>
<tr><td>T3</td><td>Submucosa, non peritonealised pericolic/perirectal tissue</td><td rowspan="2">II</td><td rowspan="2">60-83</td><td rowspan="2">40-60</td></tr>
<tr><td>T4</td><td>Other organs or structures/visceral peritoneum</td></tr>
<tr><td colspan="7">Nodal status</td></tr>
<tr><td>N0</td><td colspan="6">Not nodal disease</td></tr>
<tr><td>N1</td><td>1-3 positive regional nodes</td><td rowspan="2">C</td><td rowspan="2">C</td><td rowspan="2">III</td><td rowspan="2">40-60</td><td rowspan="2">20-35</td></tr>
<tr><td>N2</td><td>4 or more positive regional nodes</td></tr>
<tr><td colspan="7">Metastasis status</td></tr>
<tr><td>M0</td><td colspan="6">No distant metastasis</td></tr>
<tr><td>M1</td><td>Distant metastasis</td><td></td><td>D</td><td>IV</td><td><5</td><td>0</td></tr>
</table>

Table 1.1: Stages and prognosis of colorectal cancer

Prognosis is best expressed in the duration of disease free survival (Table 1.1). The pTNM system is accepted by both the Union Internationale Centre le Cancer (UICC) and the American Joint Committee for Cancer (AJCC). This system reflects the pathological and clinical stage of the cancer, whereas the Dukes' staging system omits the clinical status of the cancer and is based on only the pathological status. The Dukes' system was originally described for rectal cancer but is also applicable to colonic cancer. This system correlates well with patient survival and is easy to recall and apply and is therefore still in wide use. A disadvantage of the Dukes' system is the absence of a specific stage to address the issues of residual tumour and known distant metastases. Turnbull modified the Dukes' system by adding the Stage D for known distant metastases and locally advanced tumour found at the time of surgery (Turnbull et al. 1967). In 1981 the ACPS system was introduced in Australia following two workshops held in Brisbane, Australia. The prospectively collected data from the Concord Hospital Colorectal Cancer Project was used to validate the ACPS system. It is essentially an extension of the original Dukes' system and a simplified version of the Concord Hospital system (Davis & Newland 1982; Newland et al. 1981). In Australia pTNM and

ACPS system are recommended by the National Health and Medical Research Council (NHMRC) (Committee 2005).

1.1.9 Treatment modalities

Surgery is the main stay of several available treatment options. Oncological resection of the affected bowel is important to improve survival outcomes. There is evidence (level III evidence) that survival outcomes are better in patients whose surgeries are performed by colorectal trained surgeons (Hodgson et al. 2003; McGrath et al. 2004; Penninckx 2001; Porter et al. 1998; Schrag et al. 2002; Wibe et al. 2003). Description of the various surgical techniques is beyond the scope of this paper.

Chemotherapy is a cytotoxic systemic therapy with many adverse effects. Chemotherapy targets circulating neoplastic cells that may be growing in distant organs. Multiple randomised controlled trials (RCTs) and meta-analysis have shown significant gains in survival rates among CRC chemotherapy patients (Dubé, Heyen & Jenicek 1997; Gray 1997). Adjuvant chemotherapy offers improved survival in the order of 15% dependent on the stage of the disease at time of diagnosis. Dukes' C stage colorectal cancer patients benefit most from adjuvant chemotherapy. There is no convincing evidence to suggest that Dukes' B CRC patients benefit from the adjuvant chemotherapy (Ragnhammar et al. 2001). An early meta-analytic assessment of 17 RCTs up to December 1986 (6791 patients) compared colorectal cancer chemotherapy groups and control groups, showed a statistically significant reduction in death rates among patients receiving at least one year of 5-FU (5-Fluorouracil) based chemotherapy. Mortality OR (Odd Ratio) was 0.83 (95% confidence interval, 0.70 to 0.98), $p = 0.03$. Further analysis of the data resulted in an even greater relative reduction in death rates for those with rectal rather than colonic cancer, 38% vs. 8% respectively, with $p = 0.02$ (Buyse, Zeleniuch-Jacquotte & Chalmers 1988).

Two further meta-analyses confirmed the benefits on adjuvant chemotherapy in patients with Dukes' C CRC. When 39 RCTs between 1959 and 1993 were pooled and analysed, the five-year survival rate was more marked for rectal cancer than for colonic cancer, at 9% and 5% respectively (Dubé, Heyen & Jenicek 1997). A leading systemic review performed by The Swedish Council of Technology Assessment in Health Care in Sweden (Ragnhammar et al. 2001) included 208 publications of which 8 were meta-analyses and 162 were RCTs. The total inclusive was 126,800 patients. In patients with Dukes' C colonic cancer the combination of 5-FU and Levamisole reduced recurrence rates from 56% to 39% and

mortality from 51% to 40% after more than 5 years of follow up. Similar results could be achieved with a combination of 5-FU and leucovorin.

Oxaliplatin, Irinotecan, Raltitrexed, Oral fluoropyrimidines and Monoclonal antibody therapy such as edrecolomab and bevacizumab have challenged the traditional 5-FU based adjuvant chemotherapy. FOLFOX (FOL – Fluorouracil, F – Folinic acid, OX – oxaliplatin) was the second line therapy after 5-FU based therapy however it has now become the first line therapy in the USA and is gaining popularity as the first line therapy in Australia. The landmark MOSAIC (Multicenter International Study of Oxaliplatin/5-Fluorouracil/ Leucovorin in the Adjuvant Treatment of Colon Cancer) trial in Europe concluded that the FOLFOX regime is superior to the traditional 5-FU. It should, however, be tailored to patient co-morbidities (André et al. 2004).

NHMRC strongly recommends adjuvant chemotherapy in patients with Dukes' C level CRC based on level I evidence. Based on level II evidence, it also recommends that a subgroup of Dukes' B should consider adjuvant chemotherapy (Committee 2005).

Because of the anatomical position of the rectum, adjuvant therapy for rectal cancer takes the form of combined chemo-radiotherapy. No RCT was carried out to assess the benefits of adjuvant chemo-radiotherapy until the late 70's. Multiple overseas RCTs consistently found that post-operative chemo-radiotherapy significantly improved survival rates and reduced rates of local recurrence; The five-year survival rate increased by at least 20% and the local recurrence rate dropped by 50% (Fountzilas et al. 1999; Group, GTS 1992; GTSG 1985; Krook et al. 1991; Lee et al. 2002; O'Connell, MJ et al. 1994; Tepper, J et al. 1997; Tepper, JE et al. 2002; Tveit et al. 1997).

Can adjuvant radiotherapy alone contribute to the long term survival of patients with rectal cancer? It is understood that loco-regional recurrence is a poor prognostic indicator of survival outcomes. Biologically, it can be explained that loco-regional recurrence could lead to development of metastatic disease and hence greater likelihood of mortality. While pelvic radiotherapy alone does not confer any improvement in overall long term survival rates, it does significantly reduce the rates of local recurrence (Wolmark et al. 2000). This outcome is even more apparent in five meta-analyses involving over 8000 patients. Adjuvant radiotherapy alone reduced the rates of both loco-regional recurrence and rectal cancer related deaths. Overall long term survival rates were not observed, however, it was due to early non-cancer deaths (Buyse, Zeleniuch-Jacquotte & Chalmers 1988).

The early non-cancer deaths were directly related to the adverse effects of post-operative radiotherapy. Post-operative adjuvant radiotherapy increases morbidities and diminishes effectiveness. The rate of pelvic sepsis is also significantly higher, as well as the incidence of small bowel obstruction secondary to adhesion and long term sequelae of radiation to small bowel. Furthermore, the disabling of rectal stricture and poor anal sphincter control results in poor quality of life (Ooi, Tjandra & Green 1999).

Pre-operative radiotherapy is based on the well known fact that radiation can shrink large tumours and enhance their resectability. It was not until the late 70's that a randomised controlled trial was initiated to investigate and confirm its potential advantages in the EORTC trial (Boulis-Wassif et al. 1984). Since then, multiple meta-analyses have concluded the overwhelming advantages of pre-operative radiotherapy (Cammà et al. 2000; group, Ccc 2001; Kapiteijn et al. 2001; Martling, A et al. 2001). It is now a common practice to irradiate advanced rectal cancer (T3/4 and/or N1/2).

A more recent advance in colorectal surgery is the concept of total mesorectal excision (TME). The rectum and its lymphovascular drainage are enclosed in an envelope of thin fascia propria. Breaching of this vital envelop during pelvic dissection would result in an unacceptably high rate of loco-regional recurrence. Total mesorectal excision refers to sharp dissection in the loose areolar tissue plane between the fascia propria of the rectum and the presacral fascia, and complete excision to the pelvic floor of the mesorectum as well as its lateral borders. This technique is reserved for mid and low rectal cancers.

In1982, Heald et al. reported 5 cases of rectal cancers where the primary tumour had extended beyond lower margins of the lesion. In 3 cases, the deposits of tumour were found more than 2 cm from the proximal primary tumour. This prompted the recommendation of total mesorectal excision with at least 2 cm clear of distal margin (Heald, Husband & Ryall 1982).

The introduction and wide acceptance of total mesorectal excision led to a significant 50% reduction in the rate of local recurrence, from 14-15% to 6% in the TME group, however this practice is not based on any randomised controlled trial (Arbman et al. 1996; Havenga et al. 1999; Heald & Ryall 1986; Martling, AL et al. 2000; McCall, Cox & Wattchow 1995; McDermott et al. 1985; Nagtegaal et al. 2002; Quirke et al. 1986; Scott et al. 1995). There are critics of TME. TME results in frequent bowel motion, higher anastomotic dehiscence rates and pelvic sepsis. Most important of all, it has not been demonstrated that TME improves survival rates.

1.1.10 Conclusion

Despite improvements in surgical resection technique and advances in adjuvant and neo-adjuvant chemo-radiotherapy, long term survival and disease-free survival rates are less than 100% unless the cancers are detected at a very early stage. Prevention is the key to achieving long term survival.

1.2 Dietary fibre

Low fibre diet is considered to be one of many possible causes of CRC. This hypothesis was popularised by Burkitt in 1969, based on epidemiological evidence showing lower rates of CRC in Africa as compared to industrialised Western countries (Burkitt 1969). Africans consume high levels of fibre in their traditional diet. It was suggested that fibre reduces the risk of CRC by increasing stool bulk and reducing transit time (and hence duration of exposure of colorectal mucosa to potential carcinogens), while altering microbial composition and reducing the intraluminal pressure. With advances in molecular biology, research into the potential protective effect of a high fibre diet has shifted to the study of short chain fatty acids. The following review emphasises both the clinical relevance and the mechanisms of butyrate acid in colorectal cancer.

Dietary fibre consists of complex carbohydrates that reach the colon and is found predominantly in vegetables, fruits, grains, seeds, nuts and legumes. In Australia dietary fibre is defined by the Australia/New Zealand food authorities (FSANZ) as the fraction of the edible part of plants or their extracts, or analogous carbohydrates, that are resistant to digestion and absorption in the human small intestine, usually with complete or partial fermentation in the large intestine. The US Expert Panel on Dietary Fibre defines dietary fibre as the endogenous components of plant materials in the diet that are resistant to digestion by enzymes produced by humans (Pilch 1987). Non-starch polysaccharides and monosaccharides are the predominant components of dietary fibre. Non-starch polysaccharides include cellulose and non-cellulosic polysaccharides such as pectins, gums, hemicellulose and mucilages (Topping & Clifton 2001). In respect to colorectal cancer, it is better to classify dietary fibre as soluble or insoluble, depending on solubility in water. Examples of insoluble fibres are the majority of hemicelluloses, celluloses and lignins. Soluble fibres are found among some hemicelluloses, pectins, gums and mucilages. Sources of the soluble fibre are fruits, vegetables and cereal grains as the walls of plant cells contain 30-40% cellulose and a high proportion of pectin polysaccharides (Harris & Ferguson 1993). Insoluble fibre is found in high proportions in wheat. Heteroxylans found in high quantities in the cell wall of wheat and maize bran are non-cellulosic polysaccharides and are the bulk of the cell wall (Fincher & Stone 1986).

Soluble fibre is highly fermentable in the proximal colon and little is detected in the distal colon and rectum. In contrast, insoluble fibre resists digestion in the stomach and the small bowel, and tends to be more slowly fermented, allowing most fermentation to occur in the left colon and rectum where the majority of the colorectal cancers occur (Folino, McIntyre &

Young 1995; Kim 2000; McIntyre, A. et al. 1991; Topping & Clifton 2001). Study of the plant cell wall discovered the protective effect of lignin on the degradation of cell wall polysaccharides by commensal bacterial enzymes in the lumen of rats (Mongeau, Yiu & Brassard 1991). The presence of lignin in the cell wall of wheat bran limits the degradation of the fibre and allows a larger amount of insoluble fibre to be delivered to the left colon and rectum. Studies in both rats and human concluded that only about one third of wheat bran was degraded during transit through the colon (Mongeau, Yiu & Brassard 1991; Stevens et al. 1988). This poses the interesting question: "What is the mechanism by which wheat bran exhibit its protective role in CRC?"

1.2.1 The average level of fibre consumption

Daily dietary fibre consumption varies around the world, being highest in African countries where traditional diets are largely comprised of unprocessed food. In the Western world, the typical diet high in processed food results in a lower daily intake of dietary fibre. It is estimated that across the North American population the average daily consumption of dietary fibre is 11-13 g/day (Lanza et al. 1987) which falls short of the recommended 35 to 40 g/day. Estimates of dietary fibre intake are dependant on method of analysis and there are two common assays used, depending on the institution. The Englyst technique described by Englyst in 1988 is commonly used in the UK (Englyst & Cummings 1988). This technique depends on enzymic reactions and ethanol precipitation, the insoluble residue being analysed for non-starch polysaccharides. Non-starch polysaccharides are calculated by adding individual neutral sugars and uronic acids. Another analytic method, the AOAC (Association of Official Analytical Chemists) technique, was developed by Prosky in the USA (Prosky et al. 1988). The core component of this assay is the use of an enzymatic gravimetric technique to first degrade starch and protein before ethanol is added to achieve a final concentration of 80%. The insoluble material is then dried and weighed. It is understood that these assays underestimate the fibre content because they are based on two assumptions. First, that all starch is digested in small bowel and, second, that complex carbohydrates are completely undigested (Englyst & Cummings 1988; Prosky et al. 1988).

Discrepancies in results between the two techniques commonly used in measuring soluble fibre components of the same food type can translate to inaccurate estimates of insoluble fibre components. For example, Englyst and AOAC techniques achieved 27% and 6% respectively for the percentage of soluble fibre in respect to total fibre in soy cotyledon fibre (Lo 1990). Further adding to the complexity, these techniques measure fibre components in laboratory

conditions versus different conditions in vivo in the gastrointestinal tract. Monro (1993) reported a much lower proportion of soluble fibre in vivo in rats than estimates based on the Englyst technique. As a result, it is difficult to compare and analyse epidemiological studies in relation to the potential effects of dietary fibre on CRC.

1.2.2 Potential protective effects of dietary fibre

Increased stool bulk

First suggested by Burkitt in 1969, faecal bulking was the initial hypothesis for the potential protective effects of dietary fibre (Burkitt 1969). Faecal weight is increased by degraded and partially degraded dietary fibre. Water-holding capacities of the dietary fibre account for its properties. The soluble fibre polysaccharides such as pectins can form a gel and thus possesses a very high water-holding property (McConnell, Eastwood & Mitchell 1974; Stephen & Cummings 1979). The dietary fibre from fruits, vegetables and wheat bran has a lower water-holding capacity than the gel forming polysaccharides. These dietary fibres are comprised mainly of parenchymal cell walls and lignified cell walls of wheat bran. Lignin replaces water in the cell wall of wheat bran which acts as a hydrophobic and hence reduces water-holding capacity (Northcote 1972). Faecal bulking also reduces transit time, reducing duration of colorectal mucosa exposure to potential carcinogens. Cummings (1976) showed that consumption of 45 g of dietary fibre reduced transit time by 18 hours, from 58 hours to 40 hours.

Binding with potential carcinogens

Secondary bile acids are known to be carcinogenic in rats. These and other carcinogens such as 1,2-dimethylhydrazine (DMH) and 1,8-dinitropyrene (DNP) are found in the colon. Both in vitro and in vivo studies have demonstrated that dietary fibre binds and reduce the concentration of these carcinogens (Harris et al. 1993; Reddy, BS et al. 1987; Roberton et al. 1991; Smith-Barbaro, Hanse & Reddy 1981; Story & Kritchevsky 1976). Possible mechanisms behind this property include the action of hydrophobic lignin in the cell wall and the trapping of carcinogens in the stool. Reduction in transit time further enhances this effect.

Lowers faecal pH

Fermentation of dietary fibre results in liberation of short chain fatty acids which collectively reduce the luminal pH of the colon. Lower colonic pH has multiple implications in reducing carcinogenesis. First, lower pH increases the calcium level for binding with free bile acids, and subsequently inhibits carcinogenic effects on the colonic epithelium. Second, 7α-dehydroxylase is necessary for the conversion of bile acids to secondary bile acids and this bacterial enzyme is repressed in an acidic environment. Third, solubility of bile acids is reduced at low faecal pH, which also decreases their availability for conversion into carcinogenic secondary bile acids (Macdonald et al. 1978; Thornton 1981). Observational epidemiological studies report an association between lower faecal pH and lower rates of colon cancer. Walker and associates (1986) examined 4 ethic groups in South Africa and found that rates of colonic cancer were lower in groups with higher dietary fibre intake and lower faecal pH level.

Alters colonic microflora

Alteration of the colonic microflora by dietary fibre is another possible mechanism. Bacteria growth is supported by dietary fibre and it is possible that subsets of bacteria could be selected to proliferate while others are repressed. This would alter the composition of microbial flora. Dietary fibre can also modulate enzyme activities of the bacteria (Jacobs 1988). Such modulation alone, however, can not be considered as contributing to the protective effects of dietary fibre on colorectal cancer development as the association between colorectal carcinogenesis and bacterial enzyme activity has not been clarified.

Prevention of insulin resistance and hyperinsulinaemia

As with other organs, insulin is one of the many important growth factors for colonic epithelial cells. Colorectal cancer cells express both insulin and insulin-like growth factor-1 (IGF-1) receptors (Giovannucci 2001; Guo et al. 1992). Insulin can exert its mitogenic effects by activating the IGF-1 receptor in vitro studies. Indirect observational studies suggest patients with acromegaly have a higher risk of developing colorectal cancer. Acromegaly is characterised by hypersecretion of growth hormones and IGF-1. It has been postulated that IGF-1 and IGF-2 promote colorectal carcinogenesis by stimulating IGF-1 receptors. Diabetes mellitus also presents with hyperinsulinaemia, however the association of diabetes mellitus and colorectal cancer needs to be elucidated. Two recent large prospective studies, one in the

US and one in Europe found a statistically significant increase in risk of developing and dying from colorectal cancer in diabetics in comparison to non-diabetic subjects. In a population-based cohort study involving 153,852 subjects, Weiderpass et al. (1997) found that, compared to the general population, diabetic subjects had 40% increased risk of developing colon cancer and 60% increased risk of dying from colon cancer. Will et al. (1998) found similar results with over 1 million respondents followed up for 13 years in the US. Diabetic male and female participants had a statistically significant increase in risk (30% and 16% respectively) of developing colorectal cancer. Injection of exogenous insulin to rats in animal experiments promoted the growth of aberrant crypt foci and colonic cancer (Corpet et al. 1997; Tran, Medline & Bruce 1996). These studies confirm the causal link between hyperinsulinaemia and development of colorectal cancer and support evidence from observational studies that obesity is a risk factor for colorectal cancer.

Fermentation to short chain fatty acids

Short chain fatty acids are the product of fermentation of dietary fibre. Of particular interest is butyrate acid, which has multiple effects on colorectal mucosa. In addition to being the major energy source for colorectal epithelial cells, in vitro it stimulates colorectal epithelial cell growth in nutrient deficient conditions and also induces apoptosis and differentiation of colorectal cancer cell lines. It exerts its effects at the molecular level by inhibiting histone deacetylase. Butyrate will be discussed in detail below.

1.2.3 Where is the evidence?

The question has been asked: “Is there evidence that dietary fibre is protective against the development of colorectal cancer?” If so, what type of evidence and how strong is it? Would there be any prospective trials or ongoing prospective interventional trials? To answer these aspects, the limitations of each type of study will be discussed first, follow by a critical review of the evidence.

Population-based epidemiological studies

There are limitations in population-based epidemiological studies, and epidemiological study of dietary fibre is an especially complex process. There are multiple compounding factors, which are neither included nor excluded from the analyses. They are based on the recall ability of participants and, depending on methodology, participants can be asked to record

dietary fibre intake on a daily, weekly or monthly basis. The daily recording of dietary fibre intake would potentially reflect an accurate account, however except among the highly motivated, this would be a burden and can lead to high rates of non-compliance, dropout or inaccurate recording on repeatedly filled out questionnaires. There is also the problem of potential bias in answering questionnaires which had not been validated before use. The most significant limitation, however, is that dietary fibre intake is highly variable on a daily basis, particularly across weekends. Other aspects of dietary intake such as high fat, high protein and high calorie food must be taken into account. Classification of food types and estimation of fibre content can be confusing and non-uniform across different studies. In addition, there are limitations associated with the variety of analytical methods used in formulating fibre content. Another important limitation is that other potential anti-carcinogens present in fibre-rich foods were not measured. These would make analysis of different studies and meta-analysis difficult. The advantages of epidemiological study are the simplicity of questionnaires with daily recording of fibre intake and non-reliance on participants' long term memory.

Correlation studies look for associations between exposures and outcomes in populations rather than individuals (Hennekens & Buring 1987). Population-based correlation studies can be used to examine the association between per capita consumption of dietary fibre and the prevalence, incidence and mortality rate of CRC in a given population. They are inexpensive, can be conducted on a large scale using simple postal questionnaires and allow easy analysis and hypothesis formation. 23 of 28 correlation studies conducted internationally found that dietary fibre such as vegetables, grains, fruits and cereals had a moderate to strong protective effect against CRC. Not only did 4 studies not find any evidence that dietary fibre reduced the risk of developing CRC but, to the contrary, all but one found an adverse effect on CRC for dietary fibre (Kim 2000). Interpretation of these studies is limited by their methodologies. Some of the studies used intake of crude fibre as a guide, which can account for underestimation of total dietary intake. Compounding factors such as pre-morbid conditions, smoking status, excessive alcohol consumption, obesity, higher CRC risk in certain individuals and other unmeasurable variables were not excluded. Overall, dietary fibre is found to have a protective effect on the development of CRC.

Case-control studies

Case-control studies of dietary fibre intake have compared control groups of subjects without CRC and groups of those with CRC. There are advantages and disadvantages over the population-based correlation studies. Accuracy of participant recall of dietary fibre intake is

one significant limitation. The advantage of this type of study is that it allows potential or established confounding factors to be either included or eliminated for both groups. As subjects were often not recruited from hospital patients with the CRC, long term follow up of these patients can not be established. Furthermore, control groups were recruited from hospitals and had other conditions or diseases which can be linked to CRC. Another problem is selection bias associated with patients who did not survive long enough to show a difference.

Three meta-analyses of case-control studies conducted in the 90's concluded that high consumption of dietary fibre reduced the risk of developing colorectal cancer (Friedenreich, Brant & Riboli 1994; Howe et al. 1992; Trock, Lanza & Greenwald 1990). Trock and associates (1990) examined 23 case-control studies (but only included 16 studies in the meta-analysis) and concluded a 43% reduction in CRC risk (odds ratio, 0.57; 95% confidence interval, 0.50-0.64) when comparing highest and lowest quartiles in dietary fibre intake. They also found that protective effects were greater in studies of vegetable consumption than in those based on estimated fibre intake, with 52% reduction (OR, 0.48; 95% CI, 0.41-0.57) vs. 42% reduction (OR, 0.58; 95% CI, 0.51-0.66) respectively.

Howe et al. (1992) performed a pooled analysis of data from 13 case-control studies from 5 continents; Asia, Australia, Europe, North and South America. They extended the study by examining individual subject data from 5287 case subjects and 10,470 control subjects and pooling the data for common analysis. This method of meta-analysis has few advantages over conventional meta-analysis of published results or narrative reviews of the literature. A statistical significant result was found with $p<0.0001$. Daily consumption of at least 31 grams of fibre was found to have a 47% (OR, 0.53; 95% CI, 0.47-0.61) reduction in risk of CRC as compared with less than 10 grams of dietary fibre. This association was not affected by adjustment for total energy intake, age, sex, height, weight or body mass index. Contrary to assumptions of better protection with higher dietary fibre consumption, after adjustment for total energy intake, age and sex they found individuals who consumed 27 grams of dietary fibre on a daily basis had a 50% reduction in risk of developing CRC as compared to those who consumed less than 11 grams (RR, 0.51; 95% CI, 0.44-0.59).

Sub-analysis of the site of CRC unexpectedly showed greatest reduction in risk for rectal cancer than right and left colon by 57% (RR, 0.43; 95% CI, 0.34-0.56), 55% (RR, 0.45; 95% CI, 0.33-0.61), and 48% (RR, 0.52; 95% CI, 0.42-0.65) respectively. Males aged above 50 benefited more by consuming more than 27 grams of dietary fibre. A reduction in risk of 56% (RR, 0.44; 95% CI, 0.37-0.53) vs. 40% (RR, 0.60; 95% CI, 0.48-0.75) was found in comparisons between males and females. Those aged over 50 years had 17% relative

reduction in risk (RR, 0.49; 95% CI, 0.42-0.57) compared to those under 50 years (RR, 0.66; 95% CI' 0.43-0.99) when both consumed at least 27 grams of dietary fibre against those who consumed less than 11 grams.

The limitation here is that the study was based on the assumption that a pooled estimate could be made of heterogeneous results. Two years later, Friedenreich and team (1994) re-analysed the 13 case-control studies using a random-effects model rather than a fixed-effects model to estimate the pooled Odd Ratio. The OR were 0.46 and 0.51 (95% CI, 0.34-0.64) for using a random-effects model vs. a fixed-effects model respectively. These authors included four additional case-control studies in the pooled analysis and concluded that daily consumption of greater than 27 grams of dietary fibre led to a 50% reduction in risk of developing CRC compared to those consumed less than 11 grams per day (OR, 0.49; 95% CI, 0.37-0.65).

The protective effect of dietary fibre is also extended to development of colorectal adenomas. A number of case-control studies estimated the reduction in risk ranged from 10% to 60% in those consumed a high quantity of dietary fibre (Haile et al. 1997; Hoff et al. 1986; Macquart-Moulin et al. 1987; Neugut et al. 1993). Because of the small sample size of these studies, general conclusions can not be reached.

Can the protective effects of dietary fibre be translated into prospective studies?

Prospective studies can measure long term effects of diet across a large number of healthy subjects, include follow up over time and assess the number of cohort members who develop CRC and have a number of advantages over time-trend correlation population based and case-control studies. Selection and recording biases are minimised because diet is prospectively assessed well before development of CRC, confounding factors can be controlled and reduced, and questionnaires can be assessed and validated at regular intervals.

Similarly to the case-control studies, the majority of studies showed protective effects of dietary fibre against CRC, however measurement of dietary fibre contents again differed widely across studies. Some studies did not show protective effects and others did not follow patients long enough. A California study recruited 25,493 white California Seventh-Day Adventists and followed them up for duration of 21 years. They did not find any protective correlation between consumption of cereal or green salad and CRC (Phillips & Snowdon 1985). This was possibly due to the small quantity of fibre in the diet. A 13-year longitudinal

Japanese study which involved 265,118 healthy cohorts aged 40 and above showed an inverse relationship between dietary fibre and death from CRC. There was a 40% reduction in relative risk of dying from CRC in cohorts with daily consumption of more than 720 cm^3 of rice and wheat compared to those with daily intake of less than 180 cm^3.

Another large cohort study showed the protective effects of dietary fibre. The Cancer Prevention Study II had 1,185,125 subjects, by far the largest cohort study in the world (Thun et al. 1992). Recruited across the USA, many of the participants were friends, neighbours, family members or acquaintances of American Cancer Society volunteers. In 1982 subjects were required to answer a 4-page questionnaire about diet and other factors such as smoking, alcohol consumption, history and family history of cancer. Participants were reassessed at 2-year intervals. After 6 years of follow up, 6.7% (79,820) of participants had died, of which 1150 died from CRC. Multivariate analyses were used to compare these patients with 5746 matched control subjects drawn from the cohort. A statistically significant result was found showing that frequent consumption of vegetables and high fibre grains reduced risk of fatal CRC. Limitations of the study were a short follow up of 6 years and that participants were better educated and wealthier than the general US population. These participants would have better access to health care and this factor alone could lower CRC mortality rates. An ideal duration of follow up would be 10 years. This study is ongoing and long term follow up of cohorts is eagerly awaited.

An interesting study carried out and published in 1999 analysed data of studies from seven countries (Jansen et al. 1999). It represented 12,763 men aged between 40 to 59 from 16 cohorts in seven countries: Croatia, Finland, Greece, Italy, Japan, Serbia, Netherlands and USA. They followed the subjects over 25 years and found an inverse relationship between dietary fibre intake and CRC mortality. Based on calculations, an increase of 10 grams of daily dietary fibre intake was associated with a 33% lower 25-year CRC mortality risk. It showed that dietary fibre reduced the risk not only of developing but also of dying from CRC.

Fuchs et al. (1999) is an example of a study showing no relationship between dietary fibre intake and CRC or colorectal adenoma. They reported 787 cases of CRC in cohorts of 88,757 female participants. Over 16 years of follow up from 1980 to 1996, total dietary fibre intake showed no statically significant reduction in incidence of CRC. Median highest daily dietary fibre consumption was 24.9 grams and lowest was 9.8 grams. It is possible that daily intake of dietary fibre was too low to see any positive protective effects against CRC. There was also no high dietary fibre related decline in risk of developing colorectal adenoma in the 27,530 women who underwent sigmoidoscopy and colonoscopy between 1980 and 1994. The same

authors investigated the dietary fibre effects in men with contradicting results to the female cohorts (Giovannucci et al. 1992). Although follow up duration was only 2 years, high dietary fibre intake (highest of >28.3 g/day vs. lowest of <16.6 g/day) was found to be inversely associated with risk of developing colorectal adenoma in distal colon and rectum. Further evaluation of these cohorts at 8 years included 16,448 men and found similar results. Interestingly, it was found that soluble fibre was more protective than insoluble fibre and its effects were more significant in distal colon than the rectum (Platz et al. 1997). Another study, the Iowa Women's Health Study (Steinmetz et al. 1994), published between the Health Professional Follow-up Study and the latter publications showed a weak but statistically non-significant inverse relationship between dietary fibre intake and risk of CRC (Giovannucci et al. 1992; Platz et al. 1997). Further independent analysis of their data did not find any reduction in relative risk of CRC with increased total dietary fibre intake from either vegetables and fruits diet or vegetables or fruits alone diet.

In summary, these prospective studies produced equivocal results. The majority found protective effects of dietary fibre against CRC. Others had contradictory outcomes. In balance, however, the notion of the protective effects of dietary fibre is supported. The next question is: "Are there any randomised controlled trials to substantiate this claim?"

Intervention studies

Randomised controlled trial is a scientific means used to evaluate the effectiveness of an intervention. By far the most reliable form of scientific evidence, it involves a process of randomisation of subjects into different arms of the study and assesses the end points of intervention. Due in part to the cause and effect relationship between dietary fibre intake and CRC, randomised control trial with human subjects should provide concrete supporting evidence.

There are difficulties in designing and conducting interventional randomised controlled trials particularly in assessing the effects of dietary fibre on CRC. First of all, any RCT would require enrolment of a large number of subjects to achieve adequate statistical power and strict monitoring of dietary fibre intake is difficult to enforce. There is also a lack of molecular biological markers to indicate carcinogenesis in progress. Furthermore, because of the slow nature of carcinogenesis, long term follow up of subjects would be mandatory.

A number of strategies, however, can be employed to overcome these difficulties. High risk individuals such as those with positive family history or carrying mutated genes can be

targeted. Another strategy would be to utilise intermediate biological markers such as adenoma, proliferative index, mitotic index, and DNA aneuploidy aberrant crypts as end points. The drawback is that, except for colorectal adenoma, not all intermediate biological markers have been validated in clinical studies (Atkin, Morson & Cuzick 1992; Schatzkin, A et al. 1996; Winawer, Sidney J. et al. 1993). The limitation of colorectal adenoma is due to the fact that few adenomatous polyps progress to cancer and that only adenomatous polyps greater than 1 cm with high grade dysplastic changes on histopathological examination have the potential to become malignant. The rate of progression to carcinoma is estimated to be 2.5 polyps per 1000 per year (Winawer, S. J. et al. 1997).

The most recent review from the Cochrane Collaboration published in 2002 concedes no evidence from RCTs to support the hypothesis that increased dietary fibre intake will reduce the incidence of adenomatous polyps within a two to four years period (Asano Tracey & McLeod Robin 2002). This review included five RCTs from 1994 to 2000, with a total of 4349 patients. The four-year Polyp Prevention Trial II by Schatzkin et al. (2000) was the largest RCT and accounted for 59% in the overall analysis of the review. Three ongoing trials, CAPP 1, CAPP 2 (both examining the effects of starch) and Ishikawa et al. (1995) were excluded from the review. There are pitfalls in the studies which result in the lack of effect of dietary fibre observed in these RCTs. The common issue is that dietary fibre content was too low to exert any effect. Previous studies suggested a protective effect is seen with minimal daily consumption of 35 grams of dietary fibre. See tables 1.2 for other possible contributing factors.

- Subjects factors
    - The intervening dietary fibre intake were in efficient to exert the protective effects
    - Chemoprevention trials require a significant commitment and compliance from the subjects
    - Healthy lifestyle with little benefits from small supplementary dietary fibre
    - Enrolled subjects were already consuming above the average quantity of dietary fibre than those in the general population
    - Different type of dietary fibres

- Methodology
    - Lack of external validity of the studies due to volunteer bias
    - Validity of colonoscopic examination as small polyps can be missed

- Nature of colorectal adenomas
    - Patients with existing colorectal adenomas are more likely to develop subsequent adenomas

Table 1.2: Possible contributing factors to the failure to show any protective effects of dietary fibre in RCTs

1.2.4 Conclusion

The notion that high dietary fibre intake is protective against the development of CRC is supported by both the epidemiological and the case-control time trend studies. Due to the complexity of dietary fibre, recent review of interventional randomised controlled trials has not demonstrated its protective effects. The results of the review should be taken with caution, however, as there are ongoing interventional randomised controlled trials which are yet to be completed. More research is necessary because diet is one important factor among several which can be altered to reduce the risk of developing CRC.

1.3 Short chain fatty acids receptors: GPR43 and GPR41

The recent discovery of short chain fatty acid receptors has stimulated a surge in investigation of the role of butyrate acid in the colon. G protein-coupled receptors (GPRs) are formerly abbreviated as GPCRs. This family of receptors is a cell surface 7-transmembrane receptors. They are the largest family of cell surface mediators of signal transduction. They were also known as orphan G protein-coupled receptors because the activated ligands were not previously described. This group of receptors are the most widely exploited for therapeutic drugs intervention. Over 30% of therapeutic drugs modulate their effects via one of this super-family of receptors (Brown, Jupe & Briscoe 2005). In the human genome there are approximately 720 genes belong to the GPR super-family, 360 of which are thought to encode for sensory receptors. The other 360 are non-chemosensory receptors. Of the 360 non-chemosensory receptors, 210 physiological endogenous ligands have been found, among these the ligands for GPR 41, 43, 84 and 120 (Wise, A., Jupe, S. C. & Rees, S. 2004). The GPR43 and GPR41 receptors were identified in 1997 however it was not until 2003 that the ligands were reported (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003; Sawzdargo et al. 1997). These receptors are now un-orphanised by the innovative technique known as reverse pharmacology involving the use of a given GPR as bait and the screening of large collections of likely chemical compounds based on the known sequence of the receptor.

1.3.1 Protein sequence

GPR43 and GPR41 genes are located on chromosome 19q13.1 (Sawzdargo et al. 1997). These receptors have different sequence identities to others GPRs but share approximately 40% sequence identity with each other. Human GPR43 and 41 exhibit 52% homology to each other. They share the same endogenous ligands but not the potency. Endogenous ligands for GPR43 and GPR41 are short chain fatty acids such as acetate, propionate, butyrate and pentanoate (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003).

1.3.2 Protein pharmacology

GPR43 and GPR41 share the same endogenous ligands however the ligands do not share the same potency for the receptors. A slight difference in carbon chain length accounts for the different potencies. Among the short chain fatty acids, propionate is the most potent on both GPR43 and GPR41 receptors, while butyrate is more potent on GPR41 receptors. Acetate has more potency to GPR43 receptors (Le Poul et al. 2003). Similarly, pentanoate is more potent at GPR41 than at GPR43 in contrast to acetate which is more potent at GPR43 than at GPR41 (Brown et al. 2003).

The potency of short chain fatty acids can be measured and ranked based on the concentration-action curve of the resultant cAMP. For the GPR41, the rank order of the potency is propionate>butyrate>acetate. Acetate is 100-fold less potent than propionate and butyrate whereas propionate remains the most potent ligand for GPR43, with acetate and butyrate being equipotent (Le Poul et al. 2003). More research is needed to validate these findings as the potency of SCFAs was in contrast to those reported by Brown et al. (2005), they reported equipotency between acetate, propionate and butyrate at hGPR43, and butyrate = propionate > acetate at hGPR41 on transfected human embryonic kidney 293 (HEK293T) (Chinese hamster ovary [CHO-K1]) cell in Le Poul 2003) cell membrane. Different cell lines and techniques used in measuring intracellular calcium concentration could account for the discrepancy.

1.3.3 Mode of action

The super-family of GPR40s share similar signalling mechanisms involving both $G\alpha_i/G\alpha_o$ and $G\alpha_q$ heterotrimeric G proteins. GPR43 differs to GPR41 in that GPR43 can activate both $G\alpha_i$ and $G\alpha_q$ as compared to GPR41 which is exclusive for $G\alpha_i$. Both GPR43 and GPR41 can couple to $G\alpha_i/G\alpha_o$. These mechanisms were demonstrated in experiments with the well established Pertussis toxin-sensitive G protein. Despite differences in coupling proteins, both activate phospholipase C (PLCβ) upon the union of the endogenous ligand and receptor. The PLC β in turn increases the level of inositol 1,4,5-triphosphate (IP_3) formation from phosphatidylinositol 4,5-bisphosphate (PIP_2). IP3 then stimulates the endoplasmic reticulum to release calcium (Figure 1.3). Increase in intracellular calcium level mediates a range of cellular functions. The activated receptors also inhibit Adenylyl cyclase and subsequently increase cAMP levels. The duo process of investigating the signalling pathways of the GPR is

complex and beyond the scope of this review. A concise description is attempted, however, to aid understanding of how GPR receptors and their ligands function, and their role in CRC.

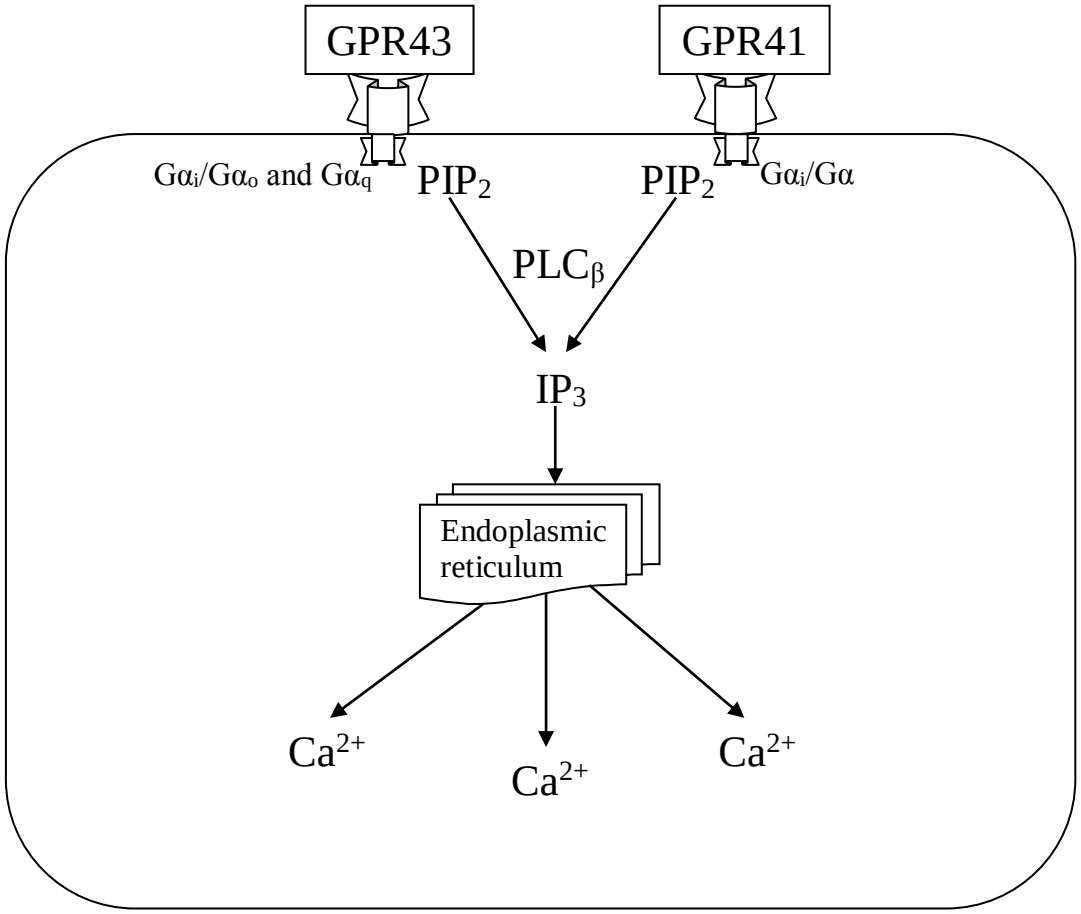


Figure 1.3: Mode of action of GPR43 and GPR41

The signalling pathways of these newly discovered receptors were derived from yeast, animal and human cell line experiments. The yeast Saccharomyces cerevisiae is an important and useful protozoa as it allows mammalian GPRs to be engineered and coupled to the endogenous yeast signal transduction pathway that responds to mating pheromone in wild-type cells. This technique can not only identify the receptors but also advance functional analysis of the receptor without a known ligand or ligands. The yeast was first transfected with a set of plasmid encoding the hGPR43, its expression and function being confirmed by the activation of the FUS1-lacZ reporter gene in the absence of any added ligand. The yeast cell expressed hGPR43 receptors were used to screen for its ligands from a pool of known GPR ligands and compounds with known or potential biological activity. Acetate was found to be among the potent ligands for hGPR43. Acetate was then used to investigate the G protein specificity of the signalling pathways. Acetate was introduced into a series of yeast strains containing different yeast/mammalian G_{α} chimeras. The induction of reporter gene

activation in the presence of acetate indicated the activation of particular G_{α} chimeras. The $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_{14}$, $G\alpha_{i1}$ and $G\alpha_{i3}$ were found to be coupling with acetate activated hGPR43. It was concluded that the GPR43 may activate the Gi, Gq and G12 families of the G proteins base on the homology of the G protein specificity for chimeras in both yeast and mammalian cells (Brown et al. 2000). Subsequent confirmation of the role of acetate in mammalian GPR43 was essential. HEK293 cells were transfected with hGPR43 and loaded with calcium sensitive fluorescent dye Fluo-4. A Fluorometric imaging plate reader (FLIPR) was employed to measure the intracellular calcium concentration upon activation of hGPR43 receptors. Elevation of intracellular calcium was a transient in response to the acetate. Pertussis toxin (PTX) is a $G_{i/o}$ family protein inhibitor. A response was not seen in PTX treated transfected HEK293 cells, indicating the signalling pathway is mediated by the $G_{i/o}$ family protein. Results again repeated and confirmed in the Xenopus laevis oocytes. Other short chain fatty acids were individually exposed to the same analysis and their potencies ranked accordingly. The protocol was applied to hGPR41 (Brown et al. 2003). Le Poul et al. (2003) contributed further and concluded that hGPR43 is coupled with both the $G_{i/o}$ and G_q proteins whereas hGPR41 is coupled with $G_{i/o}$ protein. Adenylyl cyclase was also found to be inhibited by the G-protein family, promoting the accumulation of cAMP. Inositol phosphate level is increased in the presence of activated hGPR41 and GPR43 in COS-7 and HEK293 cells. Phosphorylation of p42 and p44 mitogen-activated protein kinases (ERK1/2) also take place in CHO-K1 cells with activated hGPR41 and GPR43. The significance of the phosphorylation of ERK1/2 and its role in apoptosis is yet to be determined.

1.3.4 Tissue distribution and possible roles

GPR43

GPR43 and GPR41 expression do not share the same tissue distribution. Haematopoietic tissues such as spleen and bone marrow have the highest level of GPR43 expression (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003; Senga et al. 2003). Monocyte and neutrophil subsets account for the detecting in haematopoietic tissues of a 50-fold increase of mRNA of GPR43 in purified monocytes and neutrophils than in whole spleen tissue (Brown et al. 2003). Expression is also high in eosinophils (Nakajima et al. 2004). It was postulated that different levels of expression among monocytes reflected either the different activation stage of prepared cells, differences in the degree of differentiation or possibly a differentiation of function. A significant amount of work has been performed with SCFA and monocytes such as neutrophils, although little was known about GPR43 and GPR41 and their biological

effects. SCFAs have been implicated in induction and activation of neutrophils because of changes reported upon treatment of neutrophils in vitro with SCFAs. The changes are part of activation and phagocytosis and include calcium mobilisation, shape change, chemotaxis, and alterations in the cytoskeletal F-actin filament (Brunkhorst et al. 1992; Wajner et al. 1999). GPR43 is implicated in mediating these changes because phagocytosis activities are inhibited by Pertussis toxin (Naccache et al. 1988).

GPR43 expression was also detected in smaller quantities in non-immune tissues such as skeletal and cardiac muscle tissue and adipose, breast, placenta, lung, liver and small intestine (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003). The exact role of GPR43 in these tissues remains to be determined. Presence of GPR43 in the small intestine is speculated to be involved in the inflammatory response and possibly to mediate inflammatory bowel disease. This hypothesis is based on the fact that butyrate enema restores the mucosa in inflammatory bowel disease (Butzner et al. 1996).

GPR41

GPR41 is widely expressed in many tissues, having the highest level of mRNA expression in adipocytes (Brown et al. 2003; Le Poul et al. 2003; Xiong et al. 2004). The pancreas and spleen have the second highest level of mRNA expression, and significantly lower expressions of GPR41 are found in peripheral blood mononuclear cells, lung, placenta, liver, pituitary, brain, prostate, stomach, small intestine, kidney and bone marrow. Cardiac muscle, skeletal muscle, bone and cartilage are among tissues with negligible expression (Brown et al. 2003; Le Poul et al. 2003). Immunohistochemistry staining of the GPR41 surface receptors confirmed its presence in those tissues listed above.

The exact role of GPR41 in intestinal tissue, and indeed in many other tissues, is yet to be determined. The hypothesis that GPR41 plays an important role in energy sensing stems from high levels of its mRNA expression in adipose tissue as well as during differentiation in culture of adipogenic cell lines such as mouse 3T3 fibroblasts and Ob-Luc cells into adipocytes. Leptin release was detected upon stimulation with propionate in both in vitro and in vivo mice experiments (Xiong et al. 2001). Questions were raised as to whether this was the possible mechanism behind the significant findings of reduced appetite or dietary intake in some mammalian species when SCFA was administrated either as intravenous infusion or in the diet (Farningham & Whyte 1993; Pinchasov & Elmaliah 1995). This hypothesis, however, is far from being tested in human subjects.

1.3.5 GPR43 and GPR41 roles in colorectal cancer: The missing link

Butyrate is the most abundant short chain fatty acid (SCFA) present in the human colon and rectum. It is the end product of the fermentation of dietary fibre in the colon. The daily level of butyrate produced in the human colon and rectum is significantly high and is estimated to be above 200 mmol (Høverstad 1986). All of the butyrate and other SCFAs are absorbed by colonocytes. Butyrate has been demonstrated in vitro to have anti-neoplastic effects by inducing of $p21^{wafl/Cip1}$ expression through the inhibition of Histone Deacetylase (details in next section).

Unanswered questions remain as to whether the induction of $p21^{wafl/Cip1}$ is the only mechanism in inducing apoptosis and anti-neoplastic effects mediated by butyrate. Unaddressed are the issues of how butyrate is delivered to the cytoplasm by such mechanisms as direct diffusion or how monocarboxylate transporter 1 (MCT1) might be accountable for the delivery of butyrate necessary for the apoptotic effects. No intracellular transporters have been identified to account for the regulation of the intracellular concentration of butyrate. Little is known about the possible transporting mechanism within the nucleus. In seeking for more possible roles butyrate might have in regulating cell division and apoptosis, all possible mechanisms should be considered.

Gaining popularity is the hypothesis that butyrate is ligand to cell surface membrane GPR43 and GPR41 receptors and that butyrate can activate these receptors, initiating a chain of enzymatic cleavage rendering malignant or DNA damaged cells liable to apoptosis or halting cell division. While this hypothesis does not address all the issues mentioned above, it does provide more direct evidence of the effects of butyrate on apoptosis. More research is needed to substantiate or eliminate it.

1.4 Butyrate

Undigested dietary fibre is subjected to bacterial fermentation in the colon and rectum, of which process end products include long, medium and short chain fatty acids. The short chain fatty acids consist mainly of acetate (C2), propionate (C3), butyrate (C4) and pentanoate (C5) acids. Butyrate acid has been implicated as the most significant SCFA in protecting the colorectal mucosa against colorectal cancer.

Butyrate is a monocarboxylate with a very short half life in plasma of 6 minutes (Conley et al. 1998). All endogenous butyrate is produced in the colon by bacterial fermentation and an estimated >200 mmol of butyrate is produced per day (Høverstad 1986). Acetate, propionate and butyrate constitute 90% of the SCFA produced in the human colon. Butyrate alone accounts for about 30% of the SCFA. The luminal concentration of butyrate is maximal in the caecum, at approximately 25 mmol/kg of faecal material, progressively reduces in the distal colon and is least in the rectum. This is due to the high absorption rate by the colonic mucosa and the reduced quantity of fermentable dietary fibre (Cummings, JH et al. 1987). Absorption of butyrate can occur in three possible processes and in various parts of the colon. Paracellular diffusion of the anionic form occurs in the right colon, whereas diffusion of the undissociated form through the colonocyte lipid membrane occurs in the distal colon. The MCT1 is now widely accepted as the transmembrane protein that acts to transport butyrate in exchange for an intracellular anion (bicarbonate or hydroxide) (Mascolo, Rajendran & Binder 1991; Ritzhaupt et al. 1998; Tamai et al. 1995).

1.4.1 Physiological roles of butyrate

Butyrate has multiple physiological roles such as the inducing of proliferation, differentiation and apoptosis, and exhibits both proliferative and anti-proliferative properties.

The proliferative property

In the energy deficient state, butyrate is proliferative. It supports by the known fact that butyrate is the major substrate for oxidative metabolism of the colonocytes. Both the in vivo and in vitro experiments demonstrated the proliferative effects. Butyrate stimulated colonocytes growth in colon where the mucosa was rendered atrophic by means of parenteral nutrition, fibre free diet, elemental nutrition or faecal diversion such as ileostomy. In vitro experiments with cell lines reproduced the similar proliferative effects (Friedel & Levine

1992; Ichikawa & Sakata 1998; Kripke et al. 1989; Sakata 1987; Singh, Halestrap & Paraskeva 1997).

The anti-proliferative effects

The anti-proliferative and anti-neoplastic effects were observed in the state hyper-proliferation. These effects were demonstrated in the vitro studies, on the other hand it is an effort to prove in vivo. The anti-neoplastic effect is exerted at a variety of cellular levels (Table 1.3).

Luminal levels	Reduced via Reducing paracellualr permeability Promoting mucus secretion Enhancing epithelial restitution
Rate of DNA synthesis	Reduced in energy and substrate efficient state
DNA repair mechanism	Enhanced DNA repair
Differentiation	Induced and promoted
Apoptosis	Induced
Metastasis	Suppressed proteases and induced protease inhibitors involved in cell migration/invasion Reduced cell invasion in model systems in vitro

Table 1.3: Possible anti-neoplastic actions of butyrate, adapted from Sengupta et al. (2006)

1.4.2 Where is the evidence?

Enormous amounts of evidence on the anti-neoplastic effects of butyrate have been reported in the literature. Cell lines studies have provided insight to the complex carcinogenesis processes involved in CRC. Cell lines provide the alternative to unethical studies of CRC in human subjects. In addition, they allow investigation at the subcellular and molecular levels. Due to the short half life of butyrate, the delivery of butyrate to the human colon is limited. Obviously, extrapolation of the results stream from the vitro experiments need to be cautious as cell lines are not exposed to the same biological conditions as in the human colon.

Interpretation of indirect evidence is limited to several factors. Faecal butyrate level was measured rather than duration and concentration of colonic epithelium exposure to butyrate. Other factors include the need to rely on random faecal concentrations and the inability to measure accurately the level of butyrate as fermentation is a continuous process in the colon and rectum. A wide variation of butyrate levels produces across individual subject. Also, the measurement of butyrate levels in already developed CRC can not be used to predict its role in the early stages of carcinogenesis and colonic epithelium is exposed to much greater concentrations by in vivo than in vitro studies.

1.4.3 Mode of action

At cellular level, butyrate induces apoptosis by inhibition of histone deacetylase (HDAC) activity. Its significant action was discovered by the Ingram group when butyrate was found to increase the level of acetylates histones in cultured HeLa and Friend erythroleukemic cells (Riggs et al. 1977). The degree of histone acetylation is dependent on the activity of both histone acetyltransferases (HAT) and histone deacetylase (Boffa et al. 1978; Candido, Reeves & Davie 1978; Sealy & Chalkley 1978). The hyperacetylation state results in induction of $p21^{Waf1/Cip1}$ expression and subsequent suppression of cell cycle progression at the G1 phase.

Histone acetylation

Human DNA is packed as chromatin. Nucleosomes are the fundamental repeating units of chromatin. Each nucleosome is made up of 146 nucleotide base pairs of DNA wrapped around the histone octamer. Histone octamer is composed of a tetramer of H3/H4 and dimer of H2A and H2B (Grunstein 1990). Linker DNA connects two adjacent nucleosomes and is bound by linker histones. These repeating units allow the two metre long human genome to be compacted within a six micrometre wide nucleus. The major components of chromatin are negatively charged and the associated histones are positively charged.

A dynamic process regulates the chromatin structure by altering the structure of histones. The net activities of histone acetyltransferases and histone deacetylases regulate the transcription of the genes. HDAC removes the acetyl groups and restores the positively charged on lysine residues which then bind firmly to the phosphate backbone of the DNA resulting in prevention of transcription (Acharya et al. 2005). The hyperacetylation of histone allows the neutralisation of the positively charged of the associated histones and separation of DNA

from the histones. This process renders the DNA to become accessible for transcription by transcription factors (Figure 1.4).

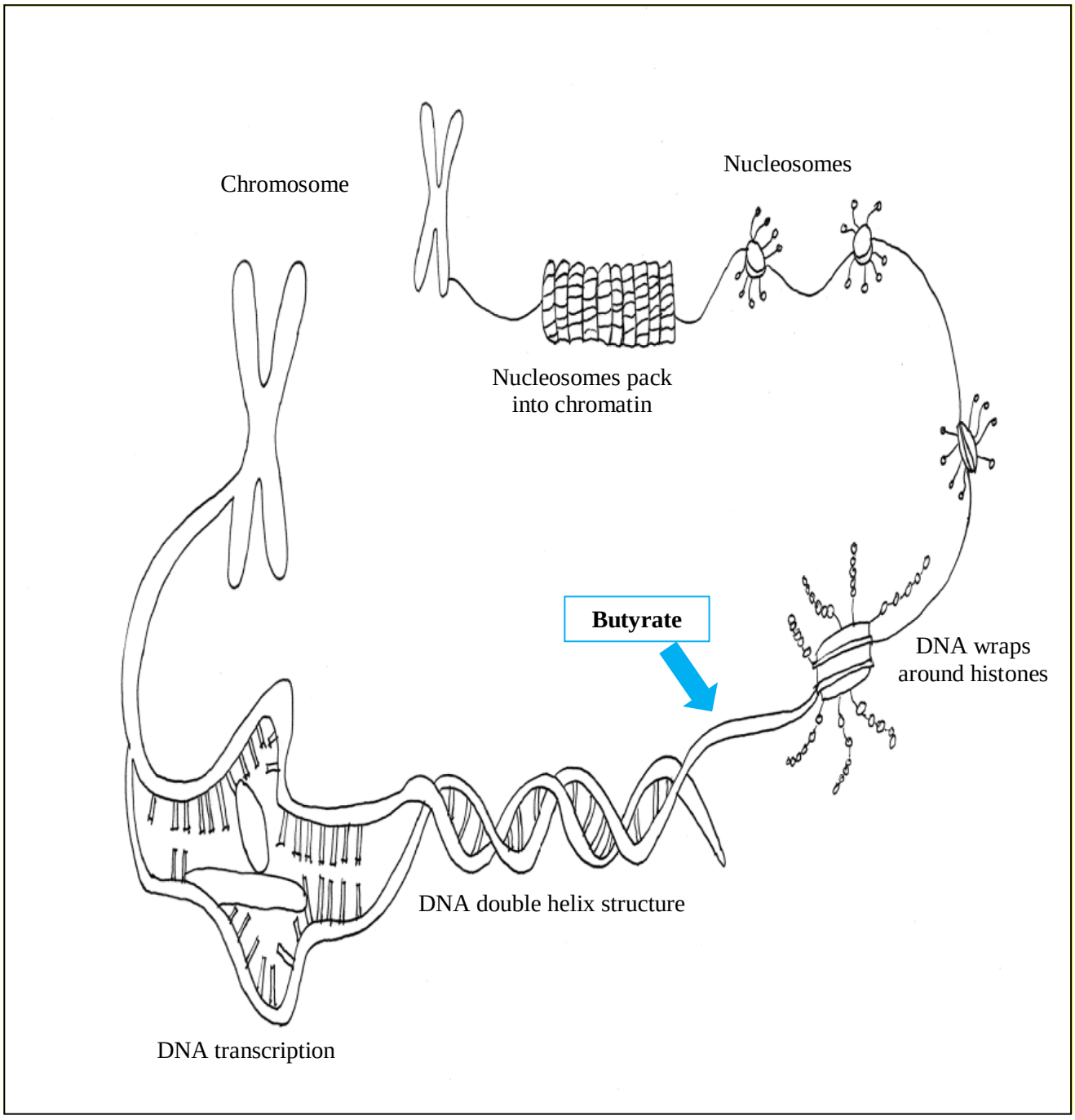


Figure 1.4: DNA and Histones

Histone deacetylases (HDACs)

18 Mammalian HDACs comprised of three classes based on the structural homologies to the three yeast HDACs (Saccharomyces Cerevisiae). Class I is analogous to the yeast RPD3, class II is analogous to HDA1 (Yeast HDAC 1), and class III is similar yeast SIR2/HST. Class I HDAC consists of HDACs 1,2,3,8 and 11 whereas class II consists of HDACs 4,5,6,7,9 and 10. Class I and II HDACs are expressed in most tissues and are primarily located in the nucleus, however class II HDACs can shuttle between the cytoplasm and the nucleus (de Ruijter et al. 2003). Unlike class I and II, class III HDACs is not inhibited by the class I and II HDAC inhibitors. Butyrate induces general histone hyperacetylation and specifically the H3 (Histone 3) and H4 (Histone 4) by non competitive inhibition of the HDAC (Sealy & Chalkley 1978). HDAC I and II are the main targets of butyrate. $P21^{Waf1/Cip1}$ gene is induced via a cis element located within 1.4 kb of the transcriptional start site, independent of p53 (Archer & Hodint 1999).

1.4.4 Histone deacetylases inhibitors

The treatment of cancer has been revolutionised since the discovery of the association between HDAC and a number of recognised cellular oncogenes and tumour suppressor genes such as Mad and Rb (Retinoblastoma protein). Comprehensive review of HDAC revealed a large number of potent inhibitors. Butyrate is one of the HDAC inhibitors first studied in the 1970s and since then numerous naturally occurring and synthetic HDAC inhibitors have been found. Of these, trichostatin (TSA) is the most potent, exhibiting activity at nanomolar concentration comparable to butyrate at millimolar concentration (Yoshida, Nomura & Beppu 1987). Butyrate, however, has the advantage as being a naturally occurring short chain fatty acid compound available in large quantities in the colorectal tract as the product of fermentation of dietary fibre. A significant number of HDAC inhibitors are in clinical trial as potential chemotherapy agents.

1.4.5 The proposed model of butyrate induced $p21^{Waf1/Cip1}$ gene expression

Gene expression is dependent on DNA promoter sequences which allow RNA polymerases to recognise initiating sequences, also known as enhancers. Subsequent products of the transcription and translation processes are proteins. Butyrate has its own response elements which allow the binding of a common transcription factor. These butyrate response elements have many different groups. The cyclin-dependent kinase 2 (Cdk2) inhibitor $p21^{Waf1/Cip1}$ is one significant groups which shares an Sp1/Sp3 binding site in the butyrate response elements (Davie 2003).

It is necessary to understand the complexity of the molecular basis of $p21^{Waf1/Cip1}$. The promoter $p21^{Waf1/Cip1}$ has six Sp1 binding sites however Sp3 is more relevant in the $p21^{Waf1/Cip1}$ promoter (Sowa et al. 1999). The Sp1 recruits HAT activity to the promoter whereas the Sp3 recruits HDAC activity. In normal physiological cells, the steady-state level of acetylated histone is low at the $p21^{Waf1/Cip1}$ promoter. The consequence is a condensed chromatin structure and no transcription. Butyrate inhibits HDAC activity, recruits the $p21^{Waf1/Cip1}$ promoter by the Sp1 and/or Sp3 and allows the HAT activity of p300 to increase the histone acetylation levels at the $p21^{Waf1/Cip1}$ promoter (Richon et al. 2000). Histone hyperacetylation unfolds the chromatin and allows transcription of the $p21^{Waf1/Cip1}$ gene.

1.4.6 The proposed model of arresting cell cycle by $p21^{Waf1/Cip1}$

There are four phases in cell division, G_1, S, G_2, and M phases. Growth and preparation of chromosomes for replication occur in the G_1 phase, whereas synthesis of DNA occurs in the S phase. $P21^{Waf1/Cip1}$ plays a significant role in cell division, particularly in the transition from the G_1 to the S phase of the cell cycle. The normal cell cycle division involves a transient increase in $p21^{Waf1/Cip1}$ expression in the early G_1 phase as the result of transient activation of extracellular signal-related kinase and Ras mitogen-activated protein kinase pathways from the mitogenic signals (Bottazzi et al. 1999). The mitogenic signals and the transient increase in $p21^{Waf1/Cip1}$ gene expression promote assembly of active cyclin D-dependent 4,6 (D1-Cdk 4,6) kinases and inhibit activity of the cyclin E-dependent 2 (E-Cdk2) kinase complex. An unknown mechanism leads to the reduction of $p21^{Waf1/Cip1}$. The sequestration of $p21^{Waf1/Cip1}$ reduces the inhibitory effect and allows activation of cyclin E-Cdk2 complex. The activated cyclin D- and E-dependent kinases are involved in phosphorylating Rb (Retinoblastoma protein). Rb hyper-phosphorylation results in disassociation of the Rb-E2F complex, allowing

transcription factor E2F to stage the coordination of transcription of genes necessary for DNA synthesis in the S phase (Davie 2003; Sherr & Roberts 1999). Butyrate induces expression of $p21^{Waf1/Cip1}$, maintaining the inhibitory effect on the cyclin E-dependent (E-Cdk2) kinase complex which subsequently arrests cell division at the G_1 phase. The arrested cell may undergo either differentiation or apoptosis (Figure 1.5).

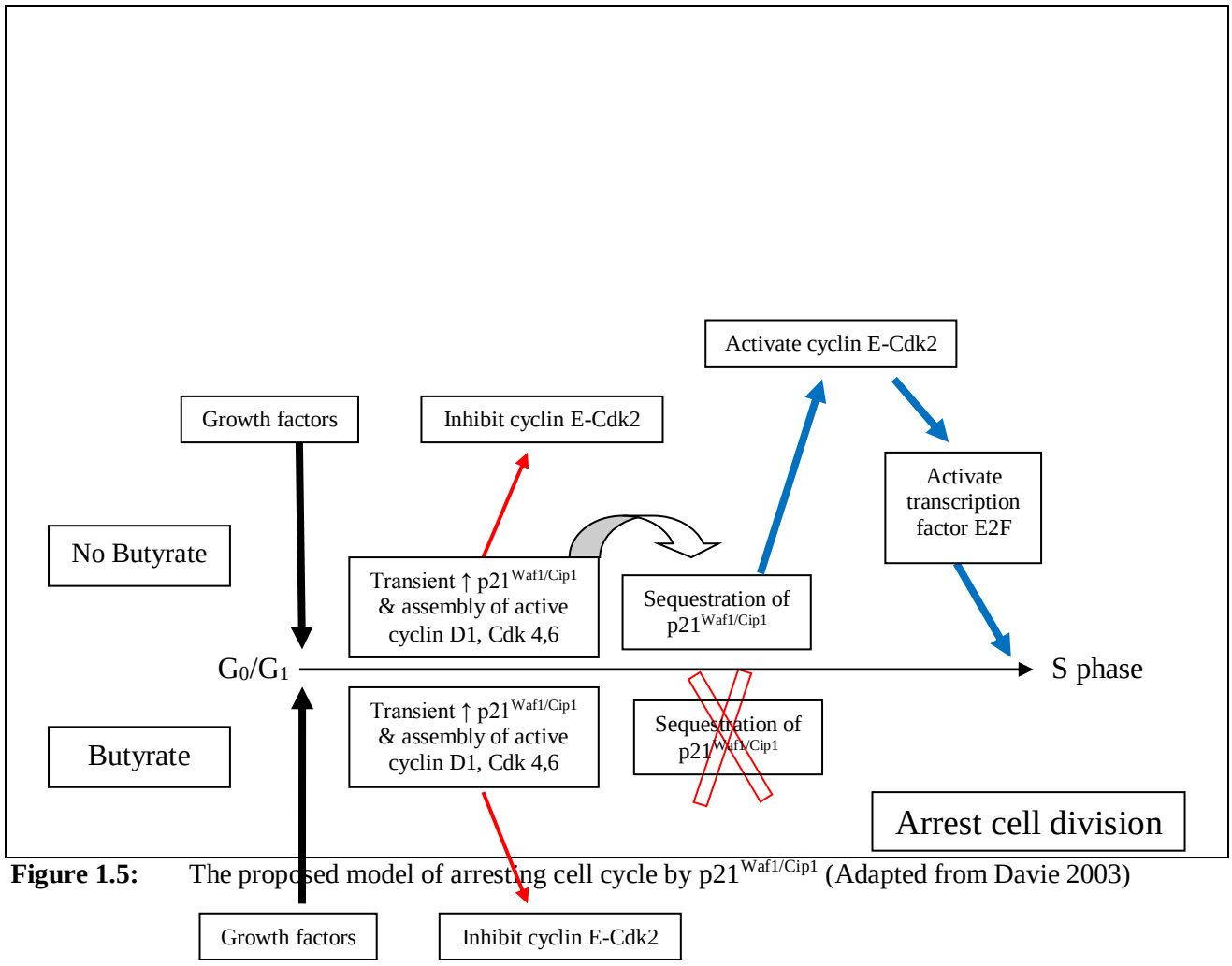


Figure 1.5: The proposed model of arresting cell cycle by $p21^{Waf1/Cip1}$ (Adapted from Davie 2003)

1.4.7 Apoptosis

In the Greek, Apoptosis is “falling or dropping off”. It is an unique mechanism of eliminating damaged cells and preventing the development of neoplastic cell. Kerr et al. (1972) was first to describe this phenomenon in 1972. This complex process is confined to an individual cell within an organ or tissue and can be mediated by two pathways: extrinsic (activation of death receptors) and intrinsic (mitochondrial mediated). The important mechanism requires meticulous balance between pro- and anti-apoptotic factors within an individual cell. A concise description of apoptosis will be included to aid understanding of cellular function and the role of dietary fibre in inducing apoptosis.

Extrinsic apoptotic pathway

The death receptor-mediated pathway is activated by the binding of the specific ligand to its corresponding cell-surface death receptor such as TNF (Tumour Necrosis Factor) receptor family, Fas or CD95 receptor and TRAIL (TNF-Related Apoptosis-Inducing Ligand) receptor. Upon the union of the ligand and its receptor, the common cellular event is initiated with the formation of intracytoplasmic complex comprising a death domain. The complex then recruits the procaspase 8 and 10 and become a supramolecular complex called death-inducing signalling complex (Figure 1.6).

Caspases are a family of cysteine proteases, they are exist in the inactive zymogens and required proteolytic cleavage by other caspases for their activation. The activation and release of active caspases allow further amplification and activation of other caspases. Caspase 8 and 10 are considered to be the initiator caspase whereas the caspase 3 is the caspase executioner.

The active caspase 8 in turn activates caspase 3, 6 and 7. It is irreversible after the caspase 3 is activated. The cell will undergo apoptosis. Activated caspase 3 in turn cleavages and activate other proteolytic enzymes targeting the cytoskeletal proteins and DNA. Proteolysis of cytoskeletal proteins and degradation of laminin in the cytoplasm cause cell shrinkage and budding whereas in nucleus, DNA condensation and fragmentation occur.

Intrinsic apoptotic pathway

The intrinsic pathway is mediated by the mitochondria. It is complex and has been undergone extensive review. The activation of the intrinsic pathway is considered as the "point of no return" in the programmed cell death. It can be activated by a range of stimuli including exogenous agents such as ionising radiation and cytotoxic agents, endogenous agents such as absence of essential growth factors or damaged DNA. The process is centred on the translocation of cytochrome c from the mitochondrial inter-membrane space into the cytoplasm (Figure 1.6).

The level of pro-apoptotic and anti-apoptotic proteins exists in the state of equilibrium. The net ratios of the proteins determine the outcomes of apoptosis. The interaction of the proteins results in the disruption of the mitochondrial membrane subsequently released and increased in the net level of free cytochrome c. Once released into the cytoplasm, cytochrome c interacts with Apaf-1 (Apoptosis-activating factor-1), adenosine triphosphate, and procaspase 9 to form the larger complex known as apoptosome. Apoptosome activates caspase 9, which in turn activates caspase 3, 6, and 7. From here the activated caspase 3 initiates the common apoptotic pathway as in the death receptor mediated. The two apoptotic pathways do communicate with each other at various levels. For examples activated caspase 8 cleavages and activates Bid protein which lead to the release of mitochondrial cytochrome c (Luo et al. 1998). Likewise, the activated caspase 6 in turn activates caspase 8 in the death-receptor pathway (Slee, Adrain & Martin 1999).

There are a number of pro and anti apoptotic proteins (Schulze-Osthoff et al. 1998). The exact nature of these proteins are to be defined, it is possible that some of these proteins are ion channels or interact with ion channels in the mitochondrial membrane, leading to increase permeability and allow efflux of the cytochrome c into cytoplasm and initiating apoptosis, see Table 1.4 (Antonsson et al. 1997; Minn et al. 1997; Schendel et al. 1997).

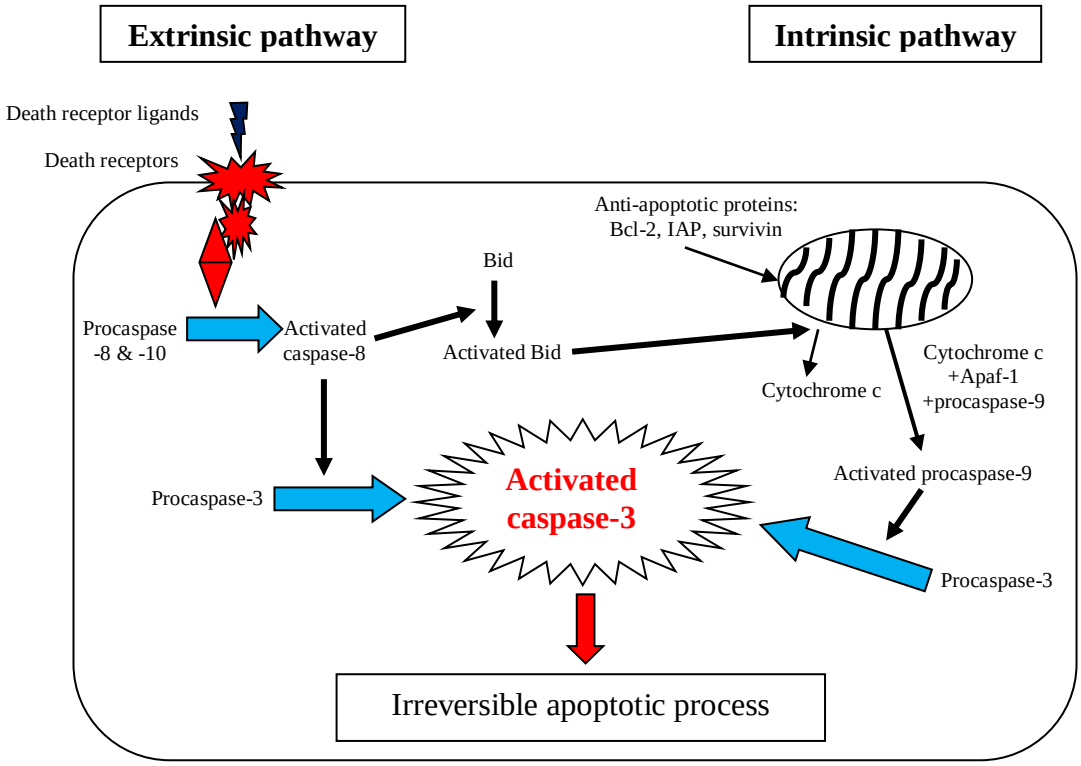


Figure 1.6: Intrinsic and extrinsic apoptotic pathways

Pro-apoptotic proteins	**Anti-apoptotic proteins**
Bcl-2 family Bax; Bcl-xS; Hrk; Bak; Bid; Bik; Bad; Bim; Hrk	Protease inhibitors NAIP; crmA; p35 Human IAP-1, IAP-2 & IAP3; Survivin Bcl-2 family Bcl-2; Mcl-1; Bcl-w; Bcl-xL; Bfl-1; Brag-1 and A2

Table 1.4: List of pro- and anti-apoptotic proteins

1.4.8 Apoptosis in colorectal cancer

In a normal colon, there is a constant dynamic process of cell renewal and disposal of senescent colonocytes. The new colonocytes are mature and migrate to the luminal surface from the colonic crypts, whereas the senescent cells are exfoliated into the colonic lumen. Apoptosis is indicated in this essential normal process. Bcl-2 is an anti-apoptotic protein and its expression is found in a decreasing trend as the colonocytes migrate upward, with minimal expression at the tip and maximal expression at the base (Hockenbery et al. 1991). Similarly, apoptosis is also evident in the development of the colorectal adenomas. Anti et al. (2001) examined colorectal mucosa biopsies from patients who underwent follow-up colonoscopy for a history of colorectal adenomas. They found apoptosis was reduced in the mucosa of patients with a history of adenomas compared to the mucosa of patients without adenoma.

CRC is the consequence of the apoptotic and proliferative processes gone wrong. There is an imbalance between apoptosis and proliferation with the pendulum swinging toward proliferation. There is extensive evidence indicating apoptosis is significantly reduced in CRC. The reduction is mediated by both extrinsic and intrinsic pathways (Greco et al. 2001; Hawkins et al. 1997; Koike 1996; Valentini et al. 1999).

The Death-receptor pathway is defective in CRC, allowing proliferation of neoplastic cells. CRC cells can adapt and escape apoptosis by down regulating of death receptors, harbouring of defective death receptors or acquiring of defective signalling pathways. Receptors are expressed on the cell surface however cells are resistant to the typical apoptotic response mediated by the immune system. In addition, some neoplastic cells can mount a counter-attack and induce apoptosis to immune cells. Fas and TRAIL are well described in CRC. FasL is a death receptor expressed by both normal cells and CRC independent of Dukes' stage (O'Connell, J et al. 1998). This receptor is non-functional in CRC and CRC cell lines. The exact mechanism is unknown but it is believed that the defect is in the signalling apparatus. Up-regulation of Bcl-2, and down-regulation of Bak, Bax, and p53 may be implicated. SW620 CRC cell line also has the ability to counter attack and induce apoptosis to immune cells by exposing intact cells to death Fas ligand (Möller et al. 1994; O'Connell, J et al. 1998).

The reverse occurs with TRAIL. TRAIL is another member of the TNF super-family. The TRAIL death-receptor pathway is mediated by unification of the TRAIL to DR4 or DR5 transmembrane receptors. Melanoma neoplastic cells are sensitive to TRAIL mediated apoptosis whereas normal colonocytes are resistant (Griffith et al. 1998; Sträter* et al. 2002).

The expression of TRAIL is an independent prognostic factor in CRC (Sträter et al. 2002). It can be concluded that CRC is more sensitive to TRAIL mediated apoptosis. The phenomenon is mediated by alternation in the signalling apparatus.

CRC accumulates a range of DNA mutations through carcinomatosis. Among other transformations such as acquisition of oncogenes and loss of tumour suppressor genes, deregulation in the mitochondrial apoptotic pathway is also necessary to continue its proliferation as neoplasm. These transformations render CRC cells more resistant to apoptosis and will be reviewed below.

APC gene

APC gene mutation occurs early in development of CRC. The APC protein has an important role in apoptosis via the Wnt pathway. The mutated APC gene results in APC protein with defective β-catenin binding sites. β-catenin is accumulated in the cytoplasm as the defective APC protein prevents the degradation of β-catenin (Senda, Shimomura & Iizuka-Kogo 2005). The β-catenin then moves into the nucleus and activates the transcription of c-myc, cyclin and Mad-1 proteins. The end result is the increase in the oronthine decarboxylase level that allows the cells to become more resistant to apoptotic stimuli (He et al. 1998).

p53

P53 is a tumour suppressor gene, its loss is common in many tumours. About 85% of CRC have mutated or non-functioning p53 gene, and its loss is occurred late in the adenoma-to-carcinoma sequence (Fearon & Vogelstein 1990). Apart from advancing tumour progression, p53 also has a role in apoptosis. In CRC, p53 can induce apoptosis either by $p21^{Waf1/Cip1}$ dependent or independent pathways. In $p21^{Waf1/Cip1}$ dependent pathway, p53 up-regulates the expression of $p21^{Waf1/Cip1}$ gene and promote cell cycle arrest at G_1 phase whereas the Bcl-2 and surviving (anti-apoptotic proteins) are repressed in $p21^{Waf1/Cip1}$ independent pathway (Hoffman et al. 2002; Saleh, Jackson & Banerjee 2000).

Other pro- and anti-apoptotic proteins

The release of cytochrome c from the mitochondria is depended on the net difference in the ratios of anti- and pro-apoptotic proteins. A number of these proteins have been identified (Table 1.4) but it is possible there are more unaccounted for. In vitro studies of CRC cell lines, the evidence is favouring the anti-apoptotic effects. The expression of pro-apoptotic proteins are increased such as Bax and Bak whereas the anti-apoptotic proteins such as Bcl-xL and Survivin are repressed (Krajewska et al. 1996; Yamamoto et al. 1999).

Apoptosis can be mediated other than by the classical extrinsic and intrinsic pathways (Bröker, Kruyt & Giaccone 2005). Studies from CRC cell lines included the HT-29 and HCT116 confirmed this mechanism does exit in the CRC. It is likely that the CRC cells can also modify their responses to overcome this mechanism as well. More research is needed to explore the potential new chemotherapy agents.

1.4.9 Role of butyrate in inducing apoptosis other than $p21^{Waf1/Cip1}$

Butyrate plays an ever increasing role in apoptosis. Apart from the proposed $p21^{Waf1/Cip1}$ pathway, it also induces apoptosis via classical apoptotic pathways. In the intrinsic pathway, butyrate induces Bak and Bax expression and represses Bcl-xL and Bcl-2 in CRC cell lines (Litvak et al. 1998; Mandal et al. 2001; Ruemmele et al. 2003). This observation was dose dependent and was seen with butyrate concentrations as low as 1 mM.

1.5 Conclusion and Aims

CRC is the third most common cancer worldwide including Australia. The incidence is increasing. The aetiology of CRC are multi-factorial involves multiple losses or gaining of tumour suppressor genes and oncogenes, also known as the adenoma-carcinoma sequence. Survival outcomes are dependent of good oncological resection and adjuvant chemotherapy. Preventing the development of CRC is the best strategy.

Low fibre diet is considered as a contributing factor to the development of CRC. Dietary fibre is complex carbohydrates found in vegetables, fruits, grains, seeds, nuts and legumes. Epidemiological and case-control studies favour the protective effects of dietary fibre against CRC. The potential effects of dietary fibre includes increased stool bulk, binding with potential carcinogens, lower faecal pH, altering colonic microflora, prevention of insulin resistance and hyperinsulinaemia, and the most significant is the fermentation to short chain fatty acids. Short chain fatty acids include acetate, propionate, butyrate and pentanoate.

The discovery of short chain fatty acids receptors, GPR43 and GPR41 in 1997 revised the interest in butyrate and its roles in the colon and rectum. GPR43 and GPR41 genes have been identified and are located on chromosomes 19q13.1, they do not share the same tissue distribution. GPR43 are highly expressed in haematopoietic tissues such as spleen and bone marrow. Monocytes and subsets of neutrophils account for the highest level of GPR43 expression. GPR41 are found in many tissues, adipocytes express the highest level of GPR41. The roles and function of GPR43 and GPR41 have not being defined, in addition their presence in human colon and rectum has not being described.

Butyrate was found to be the ligand for GPR43 and GPR41 in 2003. Butyrate is a monocarboxylate, a by-product of bacterial fermentation of dietary fibre. It is abundant in the lumen of human colon and rectum. Butyrate can induce proliferation and differentiation in energy deficient state but it can also induce apoptosis in the state of hyper-proliferation.

Butyrate has been known to mediate its apoptotic effects by inhibiting histone deacetylase activity since 1977. The inhibition of histone deacetylase results in hyperacetylation of histone, subsequently the separation of DNA from histones, allowing transcription factors access to DNA and initiating the transcription process. The advances in molecular biology in the last two decades further defined the molecular basis of butyrate. It was found that butyrate induces p21waf1/cip1 gene expression and subsequently arrests cell division at the G1 phase.

Although a model has been proposed but the exact molecular mechanism remains the subject of intense research.

Limited publications describe the presence of GPR43 and GPR41 mRNA expression in human colon and rectum. Little is known about GPR43 and GPR41 in human colorectal epithelium, in particular colorectal cancer. This thesis examines GPR43 and GPR41 via immunohistochemistry, real-time RT-PCR and colonic cancer cell lines. The hypotheses are GPR43 and GPR41 presence in normal and cancer colorectal epithelium, and that the expressions are down regulated in cancer. Butyrate down-regulates the expression of GR43 and GPR41 in colonic cancer cell lines.

Chapter Two: Learning Curve of Laboratory Techniques

Overview

Medical practitioners are not trained in laboratory research. PCR is the basis of molecular biology, real-time RT-PCR is the latest technique in semi-quantifying gene expression. This chapter emphasises the difficulties encounters and the solutions to overcome hurtles in order to achieve excellent results in real-time RT-PCR, where others have failed.

2.1 Introduction

Polymerase Chain Reaction (PCR) is the most advanced method in molecular biology to unravel and study the DNA of living organisms. It allows logarithmic amplification of short DNA sequence within a long DNA strand. The major disadvantage of PCR is an inability to quantify the number copy of DNA, as it only allows the determining of whether or not a specific DNA is present.

Real-time Reverse Transcriptase Polymerase chain reaction (real-time RT-PCR) was developed as a quantitative technique for the measuring of mRNA expression. Similar to PCR, a pair of primers is the necessary prerequisite. This process amplifies the copying of mRNA and allows comparison between differences in mRNA expression. Real-time RT-PCR is very sensitive in detecting mRNA in small RNA samples extracted from a small number of cells/cultured cells or a small amount of tissue. The quantitative process requires the use of an internal control also known as ‘internal standard’ or ‘housekeeping’ genes.

Housekeeping genes are expressed in all cells. These genes should have the same number of copies in all cells and their expression should not alter with any treatment or condition. Common housekeeping genes are usually related to cell structural proteins, common metabolic enzymes or proteins, and protein synthesis enzymes or proteins. A few examples of common housekeeping genes including Glyceraldehyde-3-Phosphate Dehydrogenase (GADPH), Beta Actin (ACTB), Major Histocompatibility Complex I (MHC I), 28S or 18S Ribosomal RNA (rRNAs) and Ribosomal Protein Large P0 (RPLP0). As no perfect housekeeping gene exists, a range of housekeeping genes should be assessed to determine that treatments or conditions do not alter their expression.

The number of copies of mRNA can be calculated as follow:

The ratio of target gene expression (experimental/control)

$$= \frac{\text{Fold change in target gene expression (experiment/control)}}{\text{Fold change in reference gene expression (experiment/control)}}$$

Control is the housekeeping gene

A standard curve is essential to calculate the number of mRNA. The standard curve is constructed from a sample with a known concentration or number of mRNA. A series of 10-fold dilutions is used such as 1/10, 1/100, 1/1000, 1/10000 and 1/100000. A minimal of 4 serial dilutions ensures more reliable and accurate results.

There are two very important parts of the thesis. The first is the optimising of RT-PCR. The second and the most crucial component is construction of a standard curve. This chapter describes the importance of experience and the steep learning curve involved in achieving the excellent real-time RT-PCR works.

2.2 Methods

2.2.1 Essential items

Real-time RT-PCR GPR43 primer assay (Detected transcript NM_005306) and GPR41 primer assay (Detected transcript NM_005304) were purchased from Qiagen, Melbourne Australia. ACTB primer assay (Detected transcript NM_001101), PMM1 (Detected transcript NM_002676) and GAPDH (Detected transcript NM_002046) were purchased from SuperArray, Sydney Australia.

2.2.2 Protocols

Tissue extraction see chapter 4
RNA extraction see chapter 4
Real-time RT-PCR reaction preparation and running protocols see chapter 4

Agarose gel electrophoresis protocol

- 2.5% Agarose gel in 1 x TAE (Tris, Acetic acid, EDTA)
- Microwave up to 2 minutes to dissolve the agarose powder in 1 x TAE
- 0.5 µg/ml of Ethium Bromide is added and mixed thoroughly before poring into the pre-made apparatus
- Mould the gel in pre-made apparatus of about of 8 mm in thickness
- Allow the agarose liquid to cool down and forming gel (30 minutes)
- Place the agarose gel in electrophoresis apparatus and fill it with 1 x TAE
- 3 µl of 0.05% Xylene Cyanol, 3% Glycerol in 0.5 x TAE add to 20 µl of PCR product solutions, gently mix by pipetting, then load 5 µl into the wells of the agarose gel
- Load TeackIt 100 bp DNA ladder
- Start the electrophoresis process for 30 minutes at voltage of 100 V and 300 mA current
- Images of the gel are developed with the UV Trans Illuminator

2.3 Results

2.3.1 Optimising real-time RT-PCR

Real-time RT-PCR is the most challenging genetic research tool ever developed. The assumptions are well published but achieving meaningful results requires expertise in optimising the mixture and setting the real-time RT-PCR machine (Bustin & Nolan 2004; Ma et al. 2006; Thellin et al. 1999). There are many techniques described in both the literature and manufacturers' guidelines. A slight variation to the setting of the real-time RT-PCR machine or the real-time RT-PCR mixture can result in negative or no products runs. Variations were made by trial and error in some cases to acquire the best products.

Different sets of primers purchased
Setting of the repeat cycle of 35x instead of 40x
Setting of the Melting temperature was crucial to eliminate the non-specific DNA products (primer/dimers errors)
Add an extra step in Melting Curve
The temperature should be set above the Tm of the non-specific products (Primer/Dimers) but it should be below 3^{o} below the Tm of the specific products. This step should be run from 5-15 seconds
Reduce the PCR primer concentration
Increase the DNA template concentration
Increase PCR annealing temperature
Hot Start PCR resulted in less primer-dimers than regular Taq polymerase PCR
A range of RNA quantity and primer solutions were tested,

Table 2.1: Techniques employed to optimise real-time RT-PCR

Variations made to progress and obtain optimisation of real-time RT-PCR (Table 2.1). The accumulated knowledge from retrospective analysis of multiple real-time RT-PCR runs and refined techniques led to success in optimisation of real-time RT-PCR. The results from a steep learning curve are displayed in table 2.2.

The optimal RNA quantity was 400 ng and primer solution of 0.6 µl For GPR43 The Primer-dimers Tm was 80^{o} PCR product of Tm was 85-85.5^{o} For GPR41 The PCR product Tm was 85^{o} The Melting temperature for both GPR43 and GPR41 primers was set at 72^{o} to optimise the Real-time RT-PCR results

Table 2.2: Steep learning curve

2.3.2 Search for appropriate annealing temperature

Primer-dimers reaction occurred in preliminary real-time RT-PCR reactions, in particular for GPR43 and GPR 41 (Qiagen, Melbourne Australia). This reaction interfered with the analysis of the results because the Rotor-Gene 6 software (Corbett research, Sydney Australia) could not differentiate between true cDNA products and primer-dimers reaction products. The technical challenge was overcome by performing temperature grading conventional PCR to establish the ideal annealing temperature of the real-time RT-PCR primers. This step was vital because the manufacturer of the real-time RT-PCR primers did not specify the annealing temperature.

Several runs with a gradient of annealing temperatures were performed to determine the optimal annealing temperature. Initial results indicated that an annealing temperature of 63^{o}C obtained the best PCR products. Further analysis and subsequent runs suggested that the optimal annealing temperature was 55^{o}C (Figure 2.1).

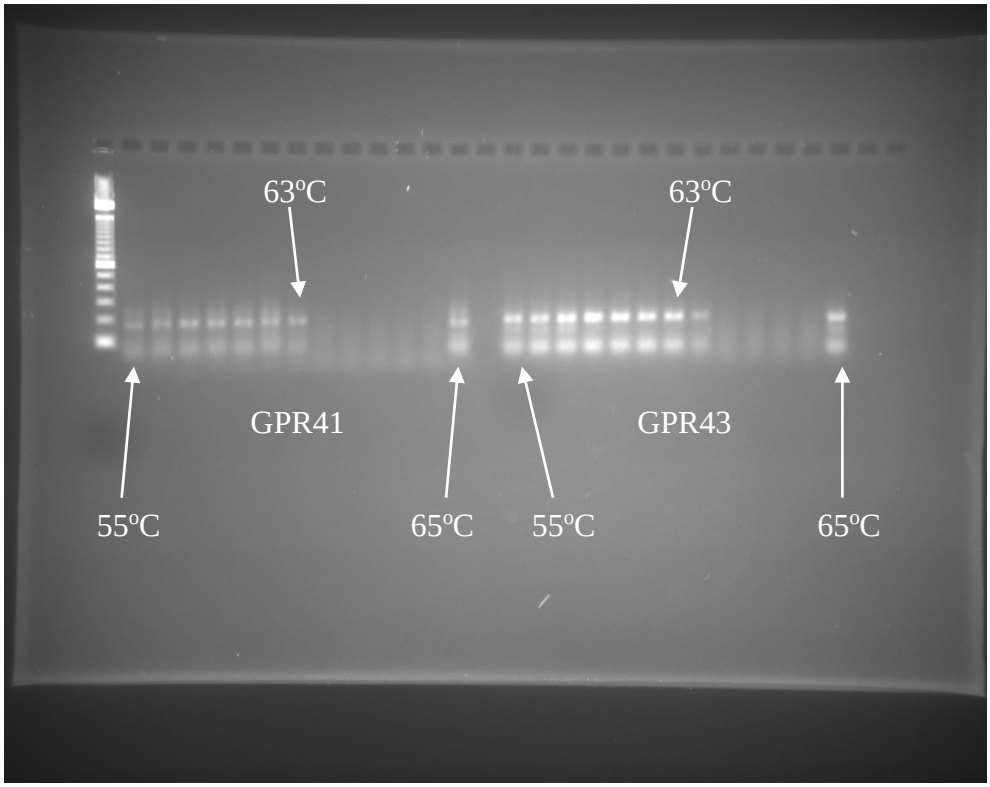


Figure 2.1: To confirm PCR products and determine ideal annealing temperature by showing the PCR products with the least non-specific band at specific temperature. The range of annealing temperature was set between 55ºC to 65ºC

2.3.3 Agarose gel electrophoresis

Following the optimisation of real-time RT-PCR, the acquired settings were tested in a trial run (setting as per real-time RT-PCR method in chapter 4). The results were highly satisfactory. The PCR products were confirmed on agarose gel electrophoresis (Figure 2.2).

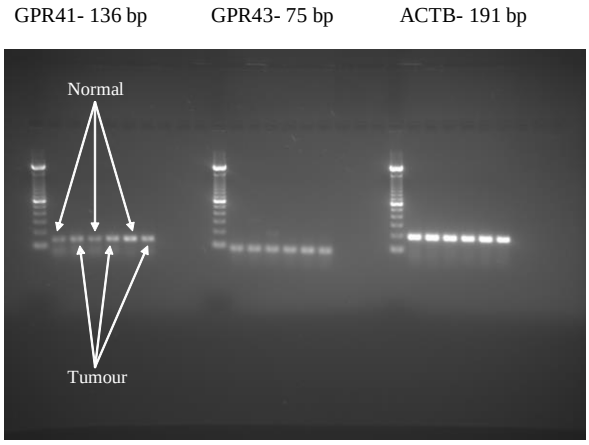


Figure 2.2: PCR products confirmed on agarose gel electrophoresis

2.3.4 Search for tissue with highest level of expression in both GPR43 and GPR41

This research was among a few pioneers assessing the mRNA expression of GPR43 and GPR41 in human colorectal epithelium and tumour. There was no commercially available or known concentrated sample from which to construct a standard curve. The search was on to find the appropriate tissue with high level expression of both GPR43 and GPR41. The level of expression of both GPR43 and GPR41 was low in normal colorectal mucosa and tumour. A number of test runs were made of tissue with a known high level of GPR43 and GPR41 expression and excellent RNA concentration in large volume of RNA solutions. The results were disappointing with no meaningful standard curve achieved (Table 2.3 and figure 2.3 – 2.6). The major obstacle was to acquire tissue with the highest level of expression of both GPR43 and GPR41.

Tissue	RNA concentration (ng/µL)	A260/230	A260/280
Sigmoid colon cancer	517.86	2.11	2.06
THP-1 cell line	520	2.12	2.06
Human adipose tissue	27.03	1.45	2.04
Human blood monocyte	62.96	1.31	2.05
THP-1 (LPS 48 hr) (Lipopolysaccharide) activated for 48 hours Human monocyte THP-1 cell line	55	2.1	2.05

Table 2.3: Tissue with a known high level of GPR43 and GPR41 expression

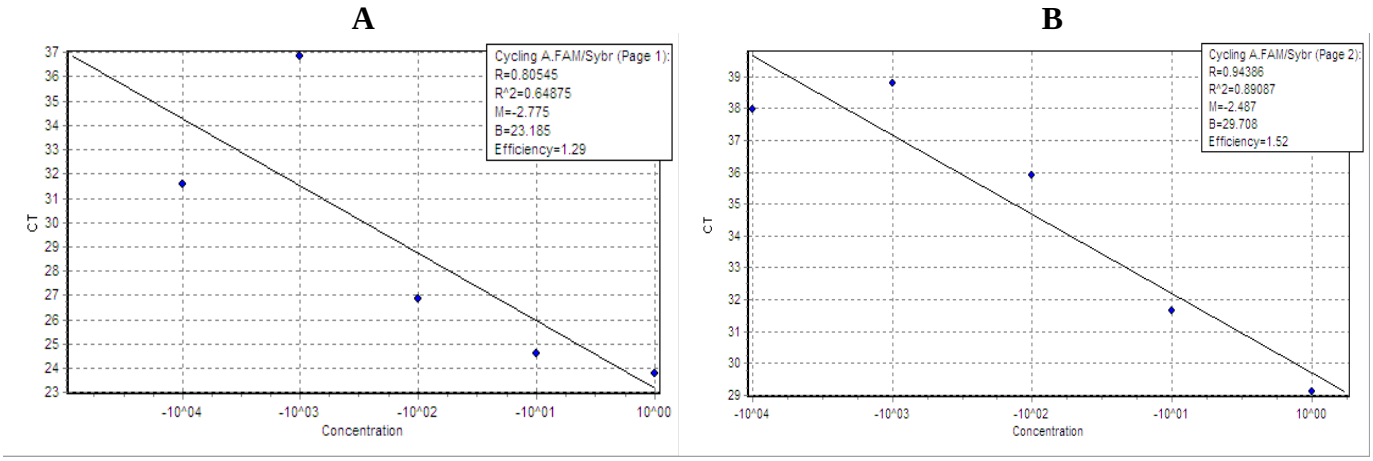


Figure 2.3: Failed GPR41 (**A**) standard curve, GPR43 (**B**) from sigmoid colon cancer RNA

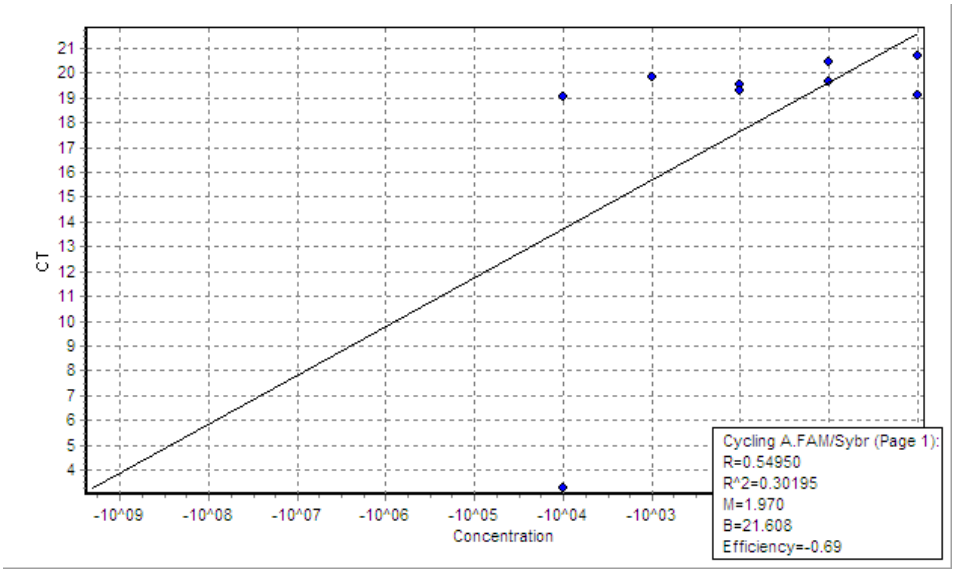


Figure 2.4: Failed GPR43 standard curve from human monocyte THP-1 cell line

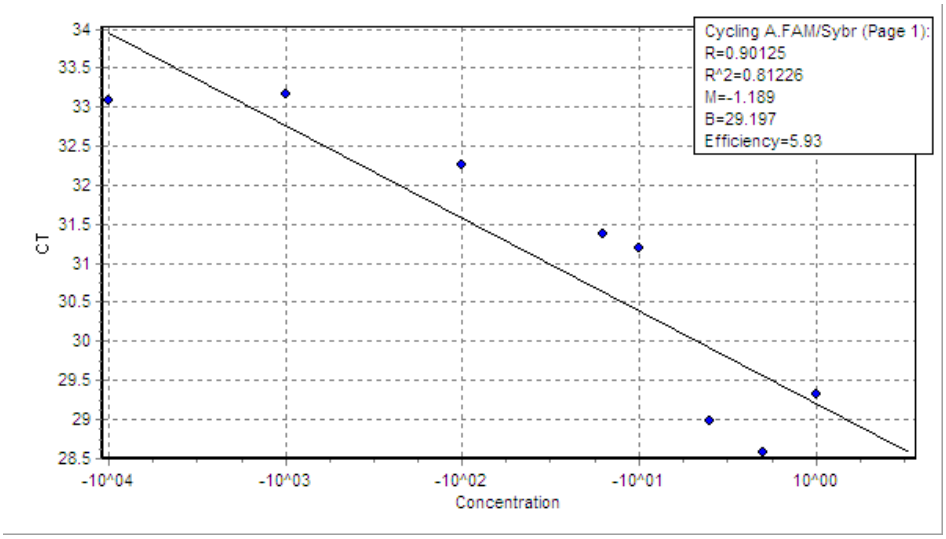


Figure 2.5: Failed GPR41 standard curve from human adipose tissue

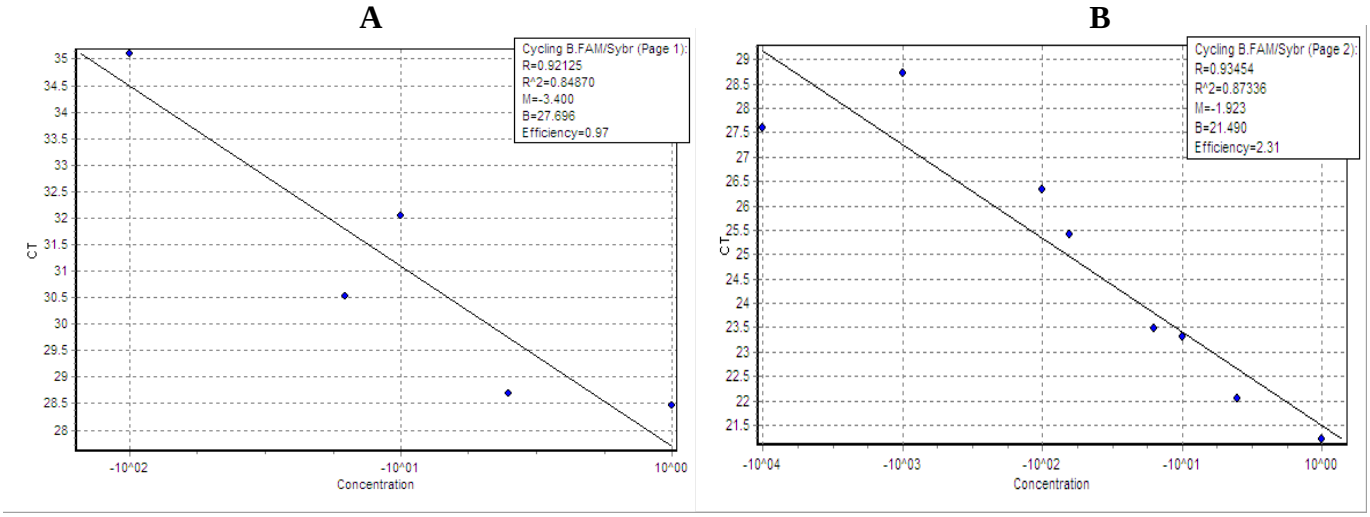


Figure 2.6: Failed GPR41 (**A**), GPR43 (**B**) standard curve from human monocyte RNA

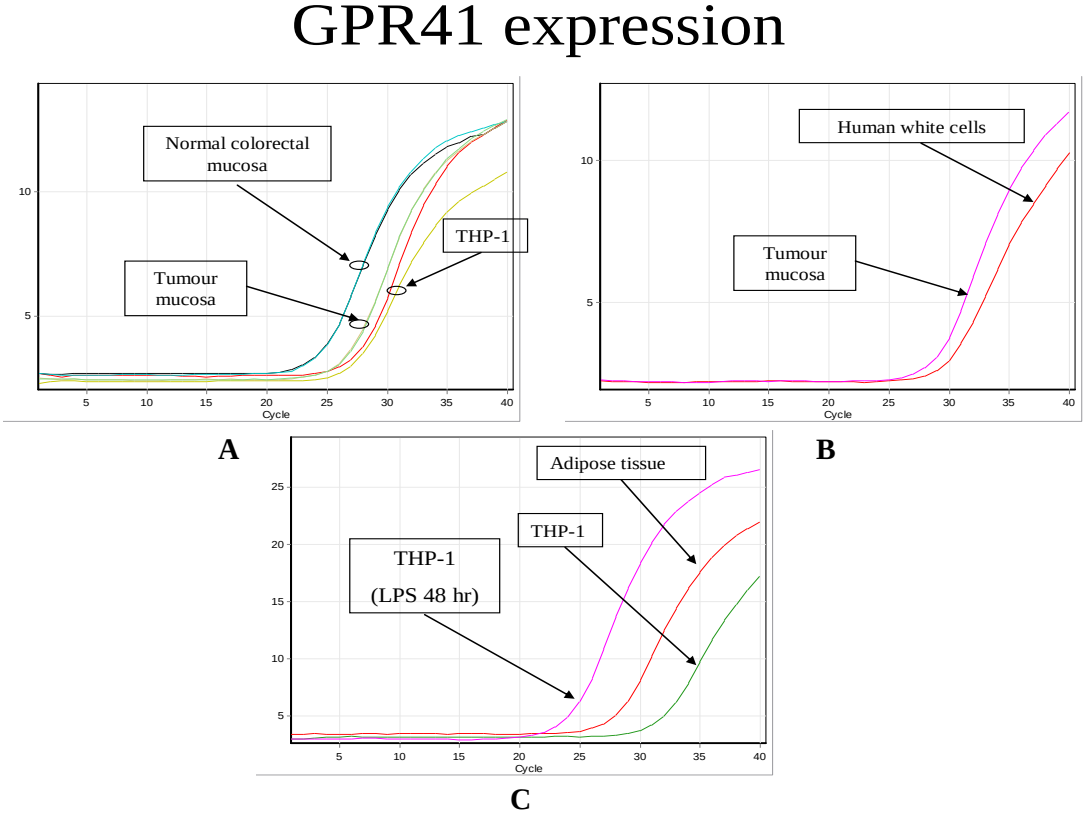


Figure 2.7: THP-1 = human monocyte THP-1 cell line, THP-1 (LPS 48 hr) = LPS (Lipopolysaccharide) activated for 48 hours human monocyte THP-1 cell line

Further reviewing of the literature suggested that the mRNA expression of GPR41 was highest in adipose tissue whereas GPR43 was highest in the monocytes (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003; Senga et al. 2003; Xiong et al. 2004). The results were far from confirming the literature but suggested that relative expression of both GPR43 and GPR41 was higher in normal colorectal mucosa samples (Figure 2.7A,B,C).

Critical analysis of results suggested that perhaps activated monocytes or macrophages rather than white blood cells would express more GPR43 and GPR41. THP-1 cell line is derived from monocyte-leukaemia hybrid cell line. THP-1 cell line closely resembles monocyte cell line. LPS (lipo-polycharride) can stimulate and induce transformation of macrophages from THP-1 cells. THP-1 cells were cultured and incubated with LPS (10µg/ml) for 48 hours. Total RNA extraction was performed and real-time RT-PCR confirmed high levels of GPR43 and GPR41 expression (Figure 2.7C).

2.3.5 Standard curve break through

Following the discovery of the highest level of GPR43 and GPR41 expression in LPS activated THP-1 cell line, LPS activated for 49 hours of THP-1 cell line was cultured and RNA solution extracted. Breakthrough in standard curve accomplished (Figure 2.8).

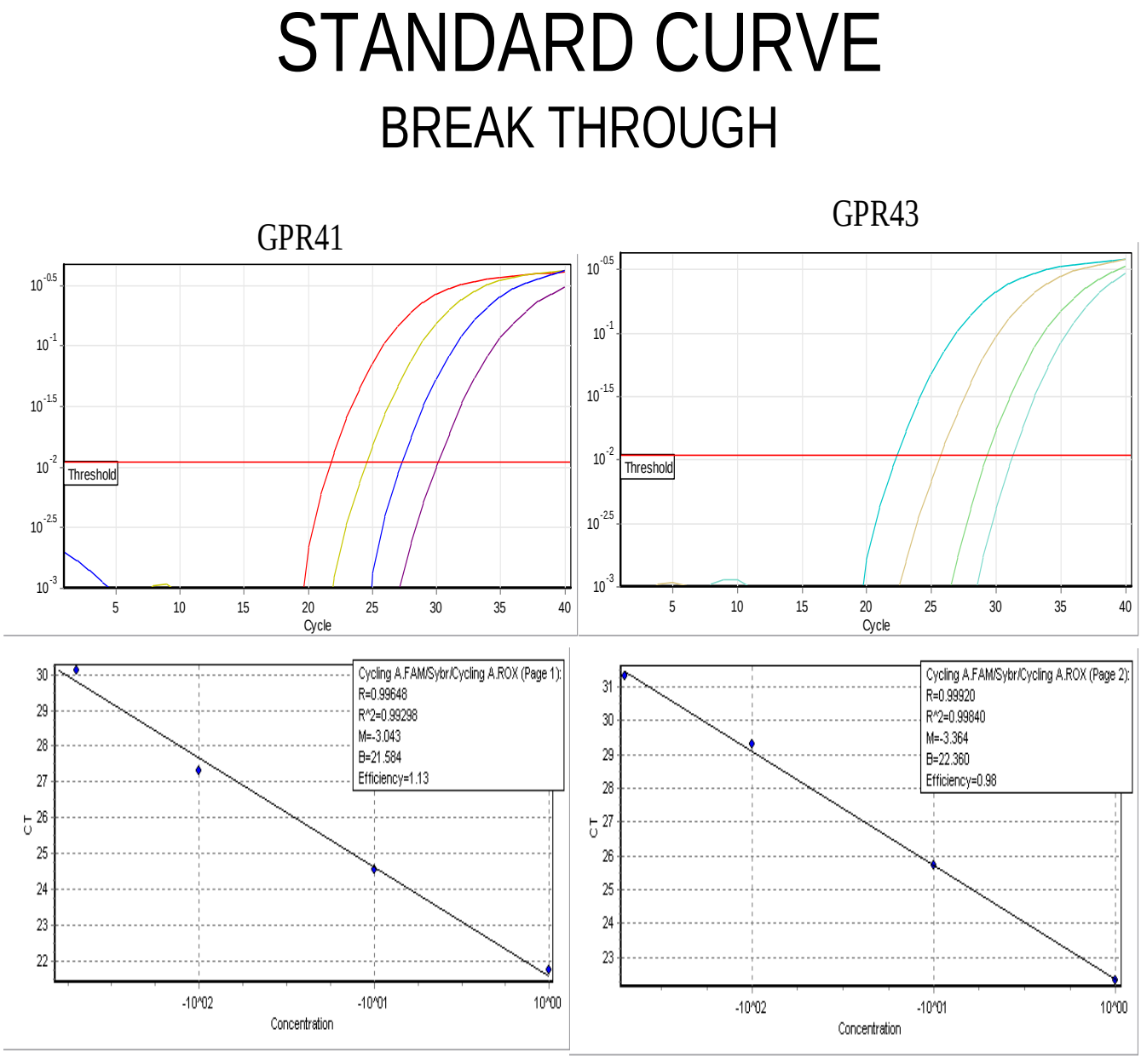


Figure 2.8: Results from Rotor-Gene 6, ideal standard curve with the R and R2 values of greater than 0.99 and the efficiency close to 1

2.4 Discussion

The search for a standard curve was the second most important part of this research. I was among a few pioneers in working with real-time RT-PCR in assessing the mRNA expression of GPR43 and GPR41 in human normal colorectal tissue and tumour. Two impediments were identified. First, neither normal colorectal mucosa nor the tumour could not be used in constructing the standard curve because levels of mRNA expression of GPR43 and GPR41 in both normal colorectal mucosa and tumour were too low. Second, there was no commercially available or (known) concentrated sample from which to construct a standard curve. In particular, the level of GPR41 expression was extremely low. Contrary to reports in the literature, I found that human adipose tissue expressed low level of GPR41 (Brown et al. 2003; Le Poul et al. 2003; Xiong et al. 2004). This stumbling block almost brought the whole research to a halt. Human monocyte cells were reported to express both GPR43 and GPR41 however the degree of expression had never been quantified. Activated monocyte cells involve in immune function including inducing cell death and phagocytosis. This led to the belief that activated human monocyte cell line may possess the same property. RNA extracted from lipopolysaccharide activated human monocyte THP-1 cell line was tested and the results were astonishing. A significantly high level of expression of both GPR43 and GPR41were recorded. This was a critical breakthrough as an appropriate sample from which to construct the standard curve was found and hence research was able to proceed. As the role of lipopolysaccharide activated human monocyte THP-1 cell line is not one of the aims, no further discussion is necessary.

Reverse transcription polymerase chain reaction is the best technique for gene expression analysis in molecular biology. Real-time reverse transcription polymerase chain reaction was designed to enhance genetic analysis by allowing quantification of mRNA expression. The advantage of one step real-time RT-PCR is that it offers rapid PCR products and hence is cost effective and efficient.

Real-time RT-PCR is a most challenging technique to master with many technical hurdles to be overcome. These range from initial RNA extraction, purification of RNA samples and optimisation of real-time RT-PCR runs to identification of appropriate housekeeping genes and construction of a standard curve. Step-by-step changes to the setting protocol required thoroughly analysis of previous runs. Of interest was the interpersonal variation in preparation for real-time RT-PCR, producing different results. Consistent results were achieved only by diligence, persistence and consistency in preparation for real-time RT-PCR. The experience

gained in the optimisation process was unique because, at times, it was almost ‘mission impossible’.

While the RNA extraction kit provided thorough instructions, the following of these instructions did not necessarily achieve a high concentrate RNA sample. Due to the limited amount of biological tissue available in this study, manufacturer’s guidelines needed to be altered to achieve the highest concentration of RNA sample. The addition of 100% Ethanol (instead of 70%) to purify RNA samples was indispensable to elimination of DNA contaminants.

Two options in real-time RT-PCR calculation are standard curve and $2^{-\Delta\Delta C}{}_T$ methods. The standard curve method generates more reliable results and is both cost and labour effective. Furthermore, it can avoid practical and theoretical problems associated with PCR efficiency assessment method (Larionov, Krause & Miller 2005; Livak & Schmittgen 2001). This method is widely used and validated in many molecular biology laboratories.

Standard curve method assumes that the housekeeping gene is not affected by testing treatment or condition (Bustin & Nolan 2004; Ma et al. 2006; Thellin et al. 1999). There are, however, a large number of housekeeping genes and not all are unaffected by treatment or condition. If the housekeeping gene is either up or down regulated by the treatment or condition then the final calculation of the tested mRNA results would change significantly. I tested three different housekeeping genes, Beta Actin (ACTB) gene, Phosphomannomutase 1 (PMM1) and Glyceraldehyde-3-Phosphate Dehydrogenase (GADPH). The level of ACTB expression was not significantly altered in the tumour tissue and hence it was used as the endogenous control (Results not shown).

Chapter Three: Immunohistochemistry of GPR43 and GPR41 in Colorectal Tissue

Overview

Immunohistochemistry allows the study of proteins in cells and tissues by using the specific labelled anti-bodies. GPR43 and GPR41 have not being reported to be present in colorectal epithelium nor colorectal cancer. The aims are to prove that GPR43 and GPR41 present in both normal colorectal epithelium and cancer, and that there is a down regulation of these receptors in cancer. 30 paraffin blocks included 15 blocks from colorectal cancer and 15 blocks from matched normal mucosa of the same 15 subjects were selected. Specific rabbit polyclonal human anti GPR43 and anti GPR41 were diluted to 1:50 and 1:100 ratio respectively. This study is the first to demonstrate the presence of GPR43 and GPR41 in human colorectal epithelium and cancer. GPR43 and GPR41 staining were positive in the cytoplasm and nucleus. Overall the positive nucleus staining was more prominent in normal epithelium than in cancer. The findings suggested a down regulation of GPR43 and GPR41 in colorectal cancer.

3.1 Introduction

In Latin Cancer means crab. Medical use of the word arose from the appearance of the cut surface of a solid tumour with the veins stretched on all sides like crab feet. At the molecular level, cancer implies aberration in both cell metabolism and cell life-cycle. Through changes in DNA, malignant cancer cells possess the ability to turn on or off genes involved in proliferation and apoptosis. This sophisticated function allows tumour cells to proliferate continuously.

Immunohistochemistry allows detection of antigens or proteins in cells and tissues by the use of specific labeled antibodies through antigen-antibody interactions that are visualised via microscopes. Interpretation of the staining slides can be semi-quantitative and is best performed by a trained pathologist. Immunohistochemistry can identify proteins or receptors and their location in cells but the function of interested proteins cannot be evaluated.

Receptor proteins can be located intra-nucleus, intra-cytoplasm or on the nucleus membrane surface**.** The roles of these receptors reflect their position. Nucleus membrane or intra-nucleus receptors have advanced proliferative and apoptotic roles in cell function. Nucleus located GPR43 and GPR41 have not been reported.

The intra-luminal concentration of butyrate is highest in the caecum and lowest in the rectum (Cummings, JH et al. 1987). Butyrate has been described as the metabolic fuel for colonocytes (Roediger 1980), and reports of its role in apoptosis have been circulating recently, but its role at the molecular level is still the subject of intense research. Butyrate acts as a HDAC inhibitor which modulates an important role in gene transcription.

GPR43 and GPR41 have been shown to be expressed in a variety of tissues but there is no report of immunohistochemistry evidence of GPR43 and GPR41 in human normal colorectal epithelium and cancer (Brown et al. 2003; Le Poul et al. 2003; Nakajima et al. 2004; Nilsson et al. 2003; Senga et al. 2003; Xiong et al. 2004). Furthermore there is no report comparing the expression of GPR43 and GPR41 between normal colorectal epithelium and cancer tissue.

GPR43 and GPR41 have been shown to be surface receptors by Sawzdargo et al. and butyrate is the ligand for these two receptors (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003; Sawzdargo et al. 1997). Butyrate induces apoptosis in animal and human cancer cell lines (Bröker, Kruyt & Giaccone 2005; Litvak et al. 1998; Mandal et al. 2001; Ruemmele et al. 2003). Indirect evidence suggests that the roles of these receptors are not clearly defined but they may be involved in cell apoptosis. Little is known about whether or not they also express and translate into proteins in human colorectal cancer. The first aim of this study is to prove that GPR43 and GPR41 exist in both normal colorectal epithelium and cancer. The hypothesis is that GPR43 and GPR41 receptors are down-regulated in cancer.

3.2 Methods

3.2.1 Subjects

30 paraffin blocks from matched 15 subjects were selected from the tumour bank. The paraffin blocks were stored at room temperature, 15 blocks from colorectal tumour tissue and 15 blocks from matched normal mucosa of the same subjects. Normal mucosa was sampled from the proximal end of the bowel specimen after surgical resection. The slides were prepared in a standard fashion via microtome at 4 µm thick.

3.2.2 Materials

Specific rabbit polyclonal human anti G protein-coupled receptor GPR43 was purchased from Affinity Bioreagents, (Colorado, USA), the dilution was 1:50. Specific rabbit human anti G protein-coupled receptor GPR41 was purchased from Phoenix Pharmaceuticals Inc (California, USA). The dilution was 1:100. Antibodies detecting system Novolink^{TM} min polymer detection system was purchased from Novocastra Laboratories (Newcastle upon Tyne, UK). A pair sample of slides with lymph node tissue as positive and negative controls.

3.2.3 Immunohistochemistry protocol

1. Dry slides in 37^{o}C oven overnight
2. Then dry slides again in 37^{o}C for 1-2 hrs before staining process
3. De-paraffinise sections in xylene (5 minutes in xylene 1 and then xylene 2)
4. Re-hydrate through graded alcohols
    a. Xylene (1) for 5 minutes
    b. Xylene (2) for 5 minutes
    c. 100% ethanol (1) for 5 minutes
    d. 100% ethanol (2) for 5 minutes
    e. 90% ethanol for 5 minutes
    f. 75% ethanol for 5 minutes
    g. Tap H_20 for 5 minutes
    h. dH_20 for 5 minutes
5. Wash slides in running water then dH_20
6. Antigen retrieval using pressure cooker (Pascal, Dako Cytomatin)

7. Submerge slides in 0.01 citrate buffer (pH 6.0) solution (in container) in 500 ml of high grade dH_20
8. Set pressure cooker at 120°C for 10 minutes, ensure a quality control strip, ensure pressure return to zero before open the lid, generally wait for 1-2 hours
9. Cool the slides down by running in tap H_20 for 5 min (alternatively)
10. Wash slides in dH_20
11. Circle the tissue with Dako pen
12. Neutralise endogenous peroxidase using Peroxidase Block for 10 minutes
13. Wash in TBS at pH 7.6 (Tris-buffered Saline) then incubate them in TBSB at pH 7.6 (Tris-buffered Saline Bovine serum albumin) for 2 x 5 minutes (different container)
14. Incubate with Protein Block for 30 minutes
15. Wash in TBS then incubate them in TBSB for 2x 5 minutes
16. Incubate with optimally diluted primary antibody over night
17. Wash in TBS then incubate them in TBSB for 3 x 10 minutes (different container)
18. Incubate with Post Primary Block for 30 minutes
19. Wash in TBS then incubate them in TBSB for 3 x 10 minutes (different container)
20. Incubate with Novolink™ Polymer for 60 minutes
21. Wash in TBS then incubate them in TBSB for 3 x 10 minutes (different container)
22. Develop peroxidase activity with DAB working solution (about 100µl per slide) for 5 minutes.
    a. DAB working solution
        i. Add 50µl of DAB Chromogen (is carcinogenic) to 1 ml of NovoLink™ DAB Substrate Buffer (Polymer)
        ii. Use within 6 hours of preparation
23. Rinse slides in water
24. Counterstain with haematoxylin for 1 minute, do it quick otherwise it will be very blue
25. Rinse slides in water for 5 minutes
26. Dehyrate, clear and mount sections
    a. Scott H_20 for few seconds, rinse in tap water then submerge in distilled water for 5 minutes
    b. dH_20 for 5 minutes
    c. 75% ethanol for 5 minutes
    d. 90% ethanol for 5 minutes
    e. 100% ethanol (1) for 5 minutes
    f. 100% ethanol (2) for 5 minutes
    g. Xylene 1 for 5 minutes

h. Xylene 2 for 5 minutes

27. Clear and mount sections
28. Interpretation and photograph of the slides

3.2.4 Interpretation of slides

Two independent pathologists (Prof Alfred Lam and A/Prof Venkatesh Shashidhar) specialising in colorectal cancer were invited to analyse the slides. Nucleus staining was assessed and degree of positive staining expressed as a percentage. Descriptive measures were used. No statistical analysis was necessary.

3.3 Results

3.3.1 Subjects

15 paired-samples from 15 subjects who underwent surgical resection between March 2004 and July 2005 were selected. These were 10 men and 5 women, with a mean age of 71.3 ± 7.46 years (58-87 years). Fifteen tumours were from different site, five from caecum, five from sigmoid and five from rectum. All tumours were well differentiated. They were all early adenocarcinoma, either in stage I or IIA. Lymphovascular invasion (LVI) were reported in 4 subjects. Only one subject (low rectal cancer) received neo-adjuvant chemo-radiotherapy (Table 3.1).

Tumour site	Age	Gender	Pathological grade	LVI	TNM	Stage	Neo-adjuvant RT
Caecum	70	F	MDC	No	T3N0	IIA	No
Caecum	69	M	MDC	No	T3N0	IIA	No
Caecum	87	M	MDC	Yes	T3N0	IIA	No
Caecum	78	F	MDC	No	T3N0	IIA	No
Caecum	72	F	MDC	No	T3N0	IIA	No
Sigmoid	76	M	MDC	Yes	T3N0	IIA	No
Sigmoid	67	M	MDC	Yes	T3N0	IIA	No
Sigmoid	61	M	MDC	No	T3N0	IIA	No
Sigmoid	74	M	MDC	No	T3N0	IIA	No
Sigmoid	77	M	MDC	Yes	T2N0	I	No
Rectal	58	F	MDC	No	T1N0	I	Yes
Rectal	75	M	MDC	No	T1N0	I	No
Rectal	66	F	MDC	No	T3N0	IIA	No
Rectal	64	M	MDC	No	T2N0	I	No
Rectal	76	M	MDC	No	T3N0	IIA	No

Table 3.1: Demographic details and pathological status of 15 subjects

3.3.2 Immunohistochemistry

For the first time GPR43 and GPR41 have been demonstrated to present in human colorectal mucosa, both in the normal and tumour (Figures 3.1A-D & 3.2A-D). The positive staining was noticed to be more prominent around the golgi apparatus, indicating true transcription of mRNA (Figure 3.4B)

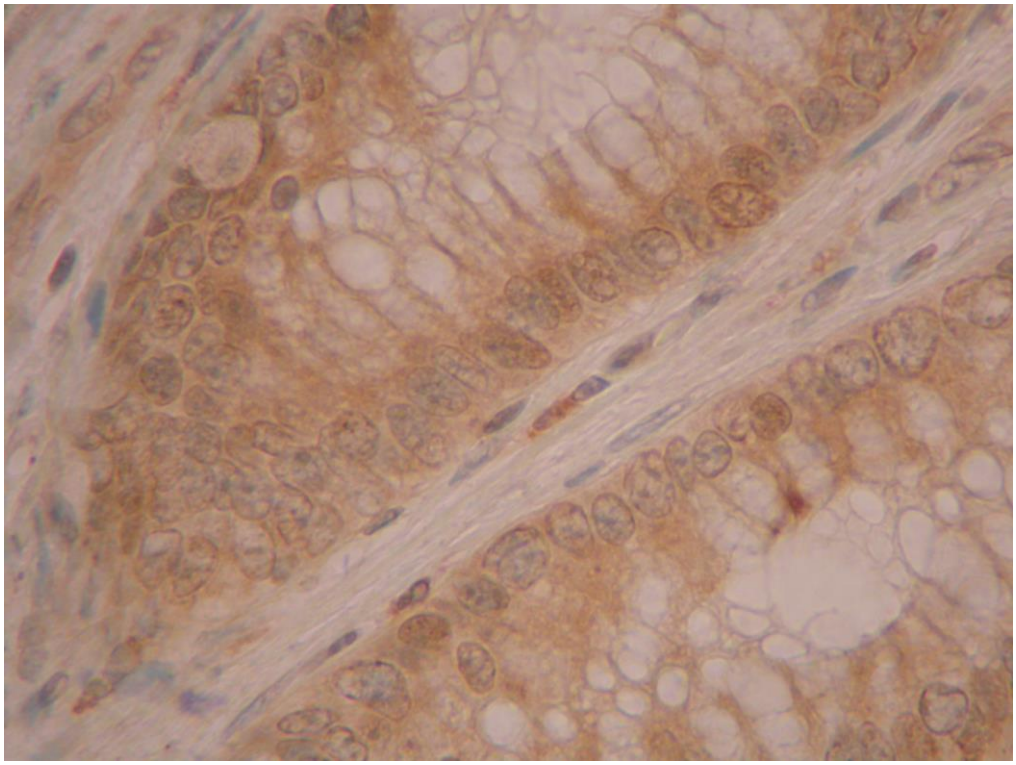


Figure 3.1A: Positive staining for GPR43 in normal mucosa

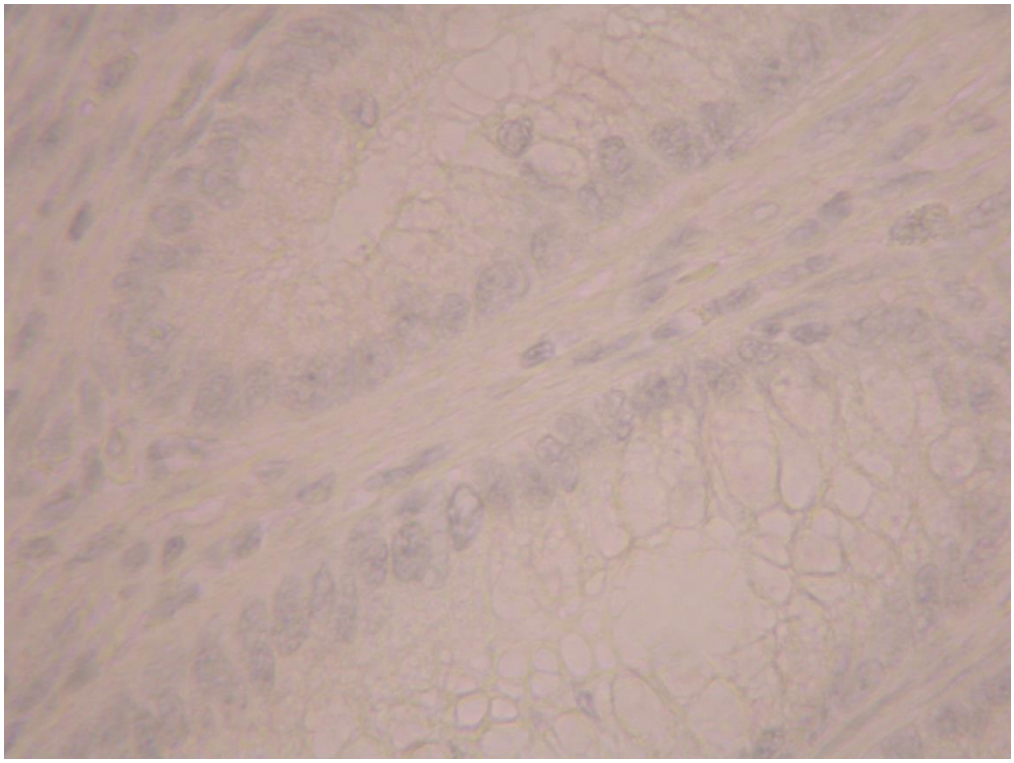


Figure 3.1B: Negative control-No primary GPR43 antibodies in normal mucosa

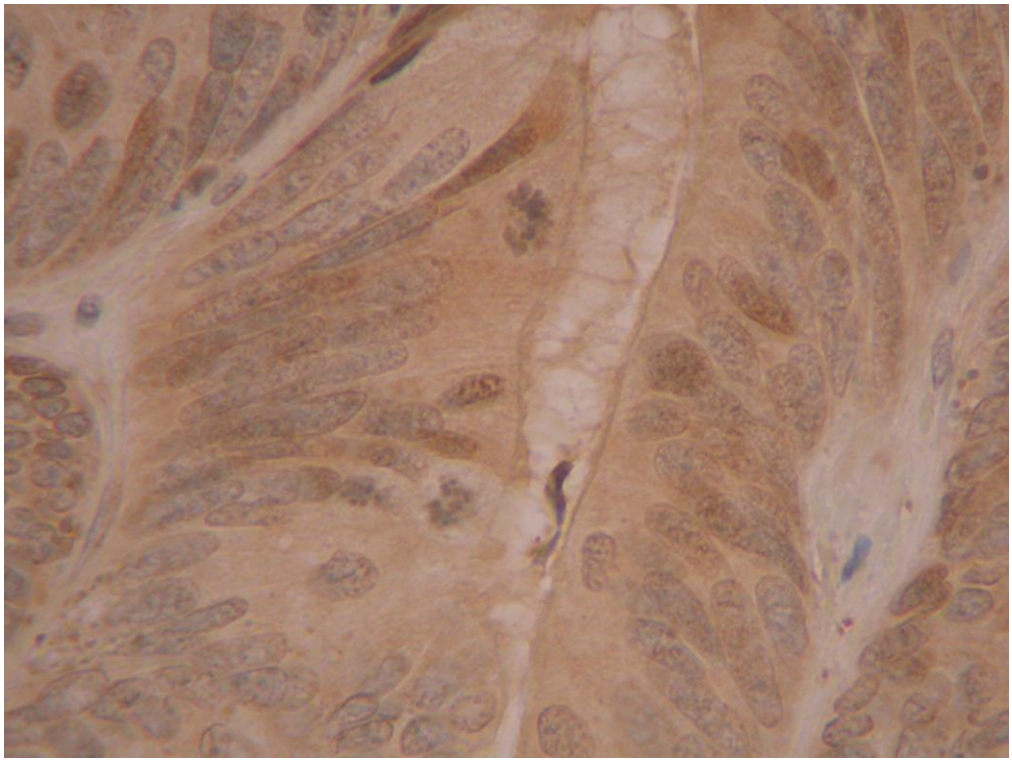


Figure 3.1C: Positive staining for GPR43 in tumour

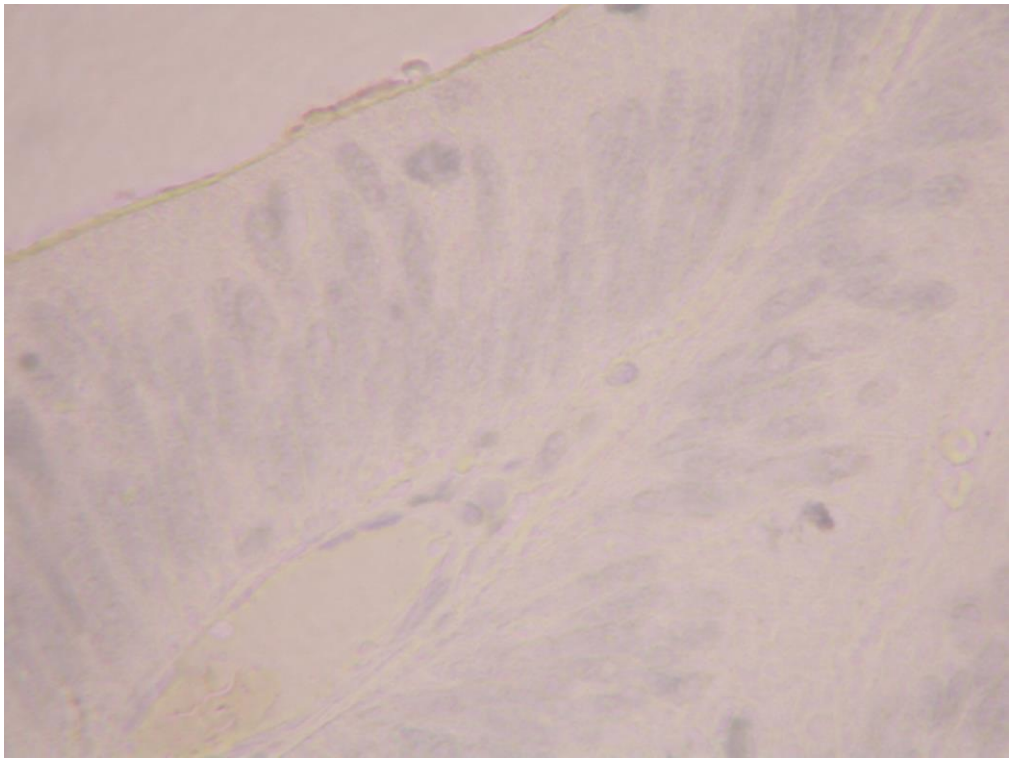


Figure 3.1D: Negative control-No primary GPR43 antibodies in tumour

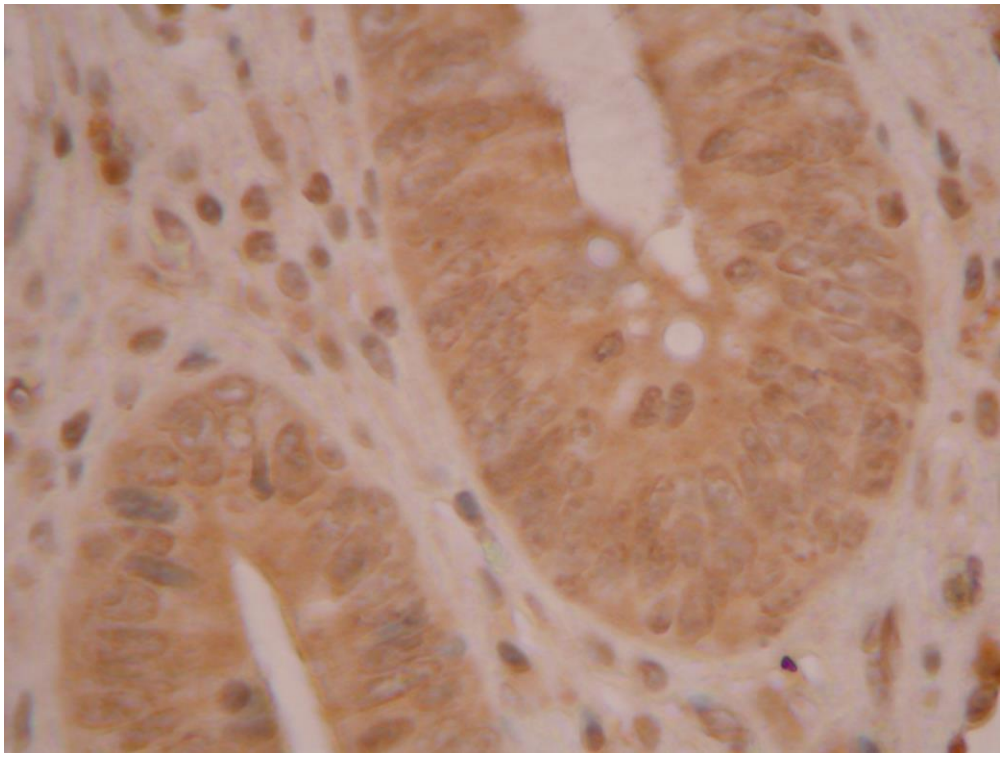


Figure 3.2A: Positive staining for GPR41 in normal mucosa

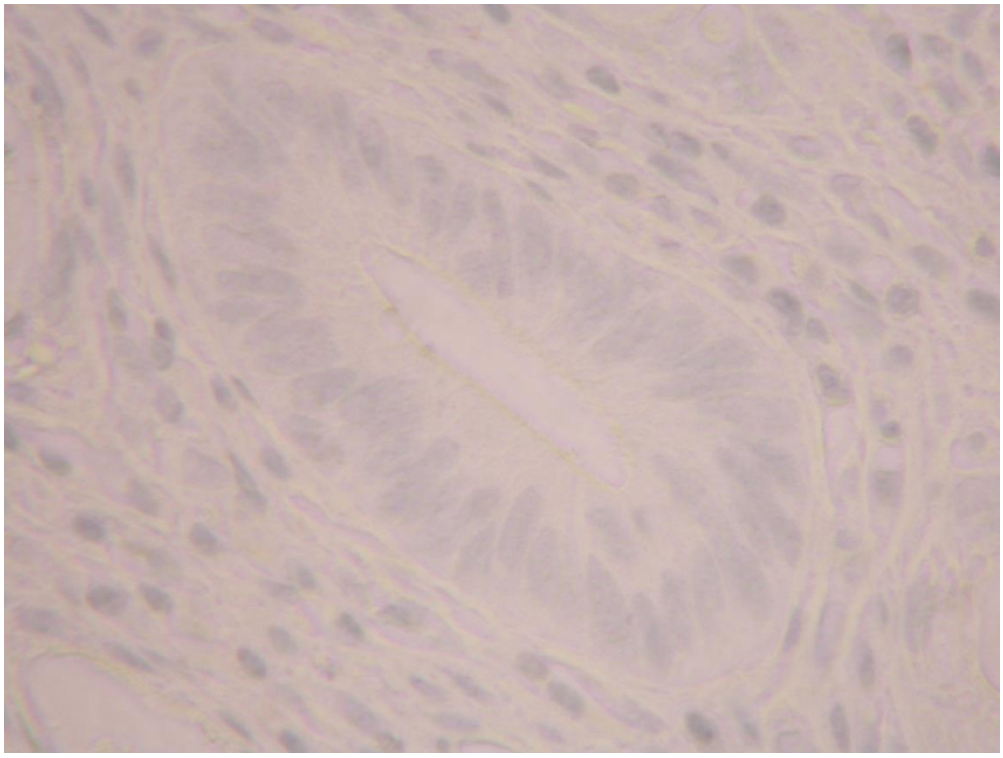


Figure 3.2B: Negative control-No primary GPR41 antibodies in normal mucosa

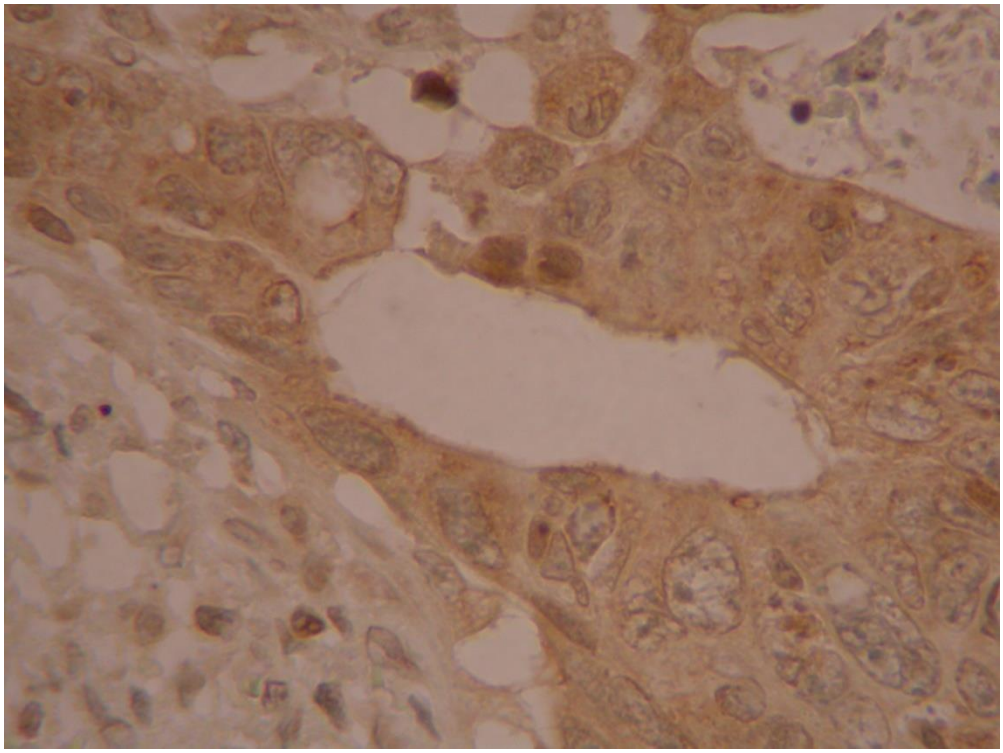


Figure 3.2C: Positive staining for GPR41 in tumour

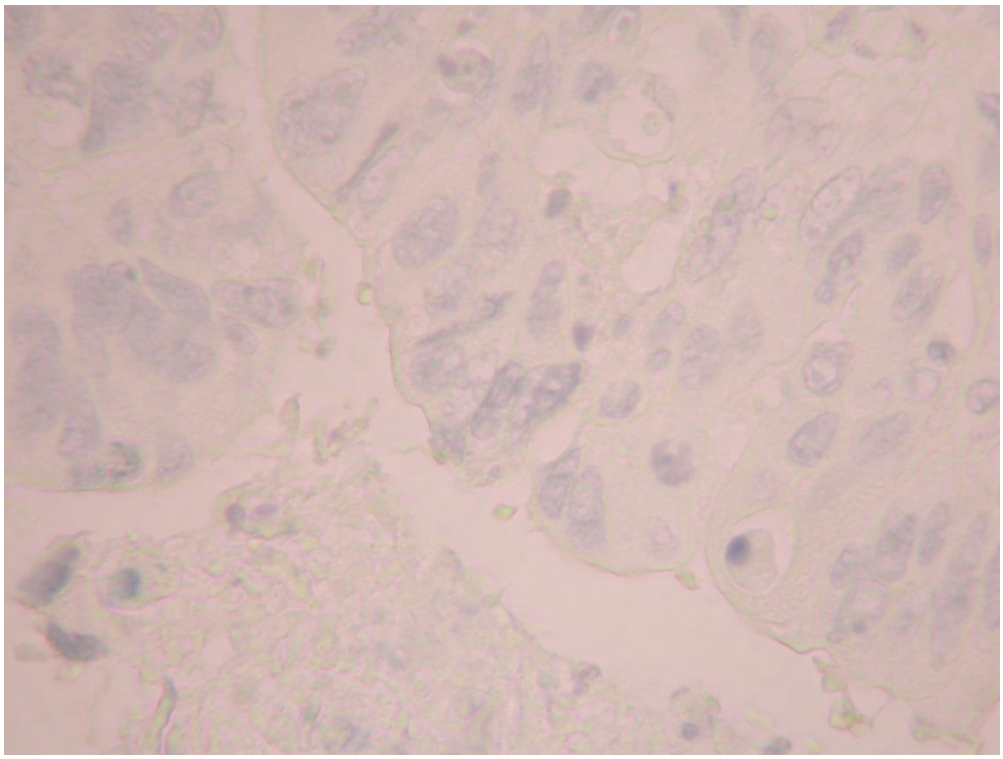


Figure 3.2D: Negative control-No primary GPR41 antibodies in tumour

3.3.3 GPR43

Both GPR43 and GPR41 showed positive cytoplasmic and nucleus staining in both tumour and normal colorectal epithelium, with a significant loss of nucleus staining for GPR43 in the paired match tumour. Of interest was the absence of any GPR43 staining in normal rectal epithelium. There was no significant difference in the degree of positive staining in tumour rectal epithelium compared to tumour from caecum and sigmoid colon. In normal colorectal epithelium, there was a trend toward decrease in degree of nucleus staining toward the rectum. This observation was not demonstrated in the tumour. These changes suggested a down regulation of GPR43 expression in tumour (Table 3.2 and figures 3.3A & 3.3B).

Site	Normal GPR43	Tumour GPR43	GPR43 remarks	LVI
Caecal	20% nuclei	3% nuclei	N > T	No
Caecal	20% nuclei	1% nuclei	N > T	No
Caecal	20% nuclei	1% nuclei	N > T	Yes
Caecal	80% nuclei	1% nuclei	N > T	No
Caecal	80% nuclei	10% nuclei	N > T	No
Sigmoid	20% nuclei	1% nuclei	N > T	Yes
Sigmoid	20% nuclei	1% nuclei	N > T	Yes
Sigmoid	negative	0% nuclei	N = T	No
Sigmoid	80% nuclei	1% nuclei	N > T	No
Sigmoid	negative	10% nuclei	T > N	Yes
Rectum	negative	1% nuclei	T > N	No
Rectum	negative	0% nuclei,	N = T	No
Rectum	negative	5% nuclei	T > N	No
Rectum	negative	5% nuclei	T > N	No
Rectum	negative	10% nuclei	T > N	No

Table 3.2: The degree of positive staining of GPR43 in colorectal mucosa, N = Normal mucosa, T = Tumour tissue

3.3.4 GPR41

As for GPR41, nucleus staining was significantly more prominent in normal epithelium than in the paired match tumour. In contrast to GPR43, there was a trend toward increase in nucleus staining towards the rectum, the nucleus staining was least in the caecum. The degree of nucleus staining in tumour epithelium was scant with slightly more evidence in the rectum than in caecum. Similar to GPR43, there was a significant down regulation of GPR41 expression in tumour (Table 3.3 and figures 3.4A & 3.4B).

Site	Normal GPR41	Tumour GPR41	GPR remarks	LVI
Caecum	80% nuclei	10% nuclei	N > T	No
Caecum	20% nuclei	0% nuclei	N > T	No
Caecum	20% nuclei	1% nuclei	N > T	Yes
Caecum	20% nuclei	5% nuclei	N > T	No
Caecum	20% nuclei	10% nuclei	N > T	No
Sigmoid	80% nuclei	1% nuclei	N > T	Yes
Sigmoid	80% nuclei	1% nuclei	N > T	Yes
Sigmoid	80% nuclei	5% nuclei	N > T	No
Sigmoid	80% nuclei	5% nuclei	N > T	No
Sigmoid	80% nuclei	20% nuclei	N > T	Yes
Rectum	20% nuclei	5% nuclei	N > T	No
Rectum	80% nuclei	0% nuclei	N > T	No
Rectum	80% nuclei	5% nuclei	N > T	No
Rectum	80% nuclei	5% nuclei	N > T	No
Rectum	80% nuclei	10% nuclei	N > T	No

Table 3.3: The degree of positive staining of GPR41 in colorectal mucosa, N = Normal mucosa, T = Tumour tissue

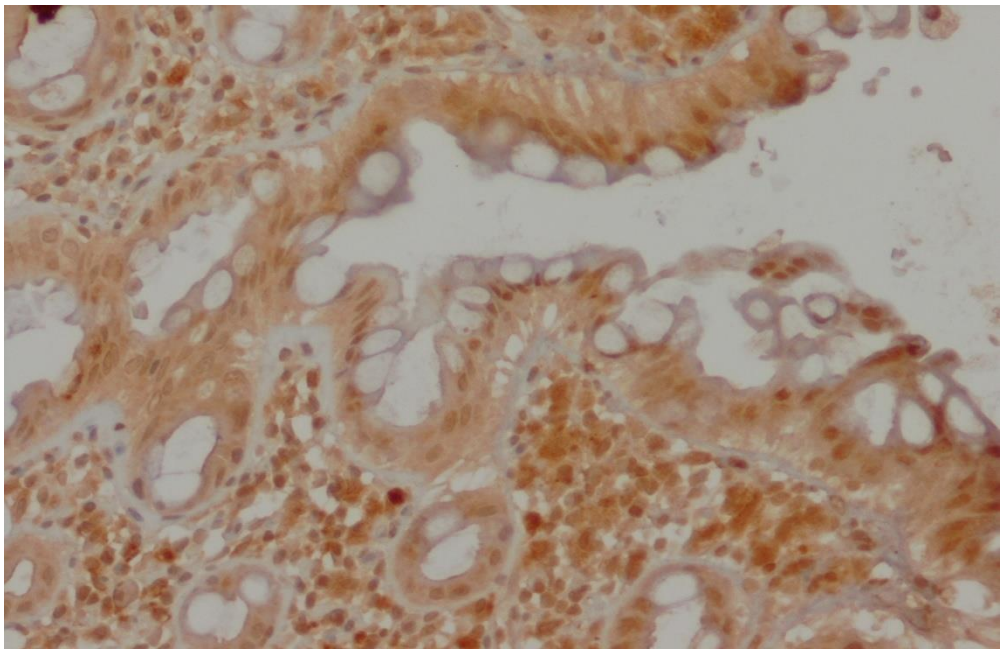


Figure 3.3A: Positive GPR43 nucleus staining in normal mucosa

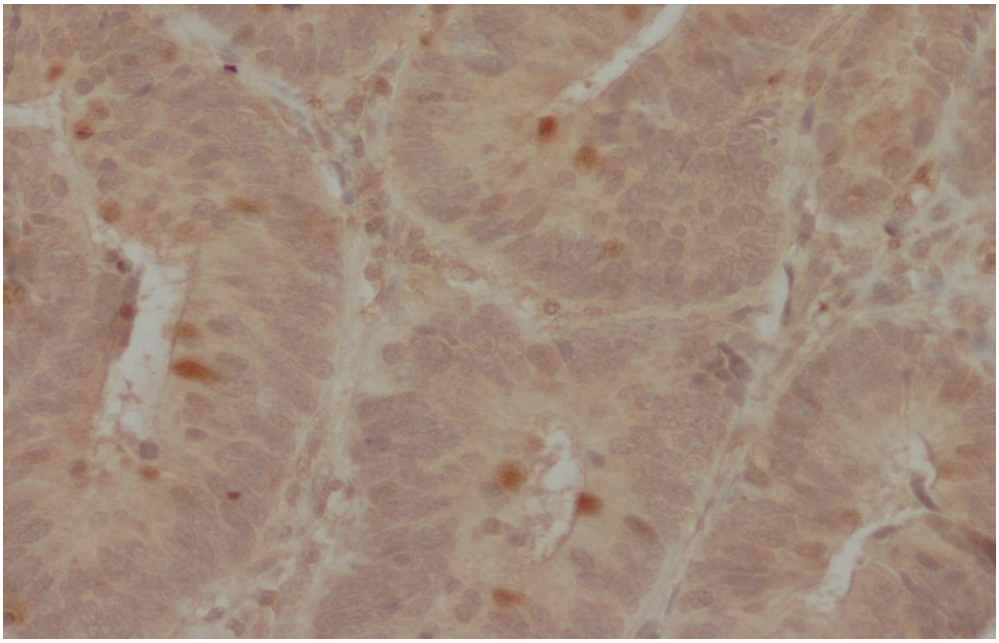


Figure 3.3B: Loss of GPR43 nucleus staining in tumour

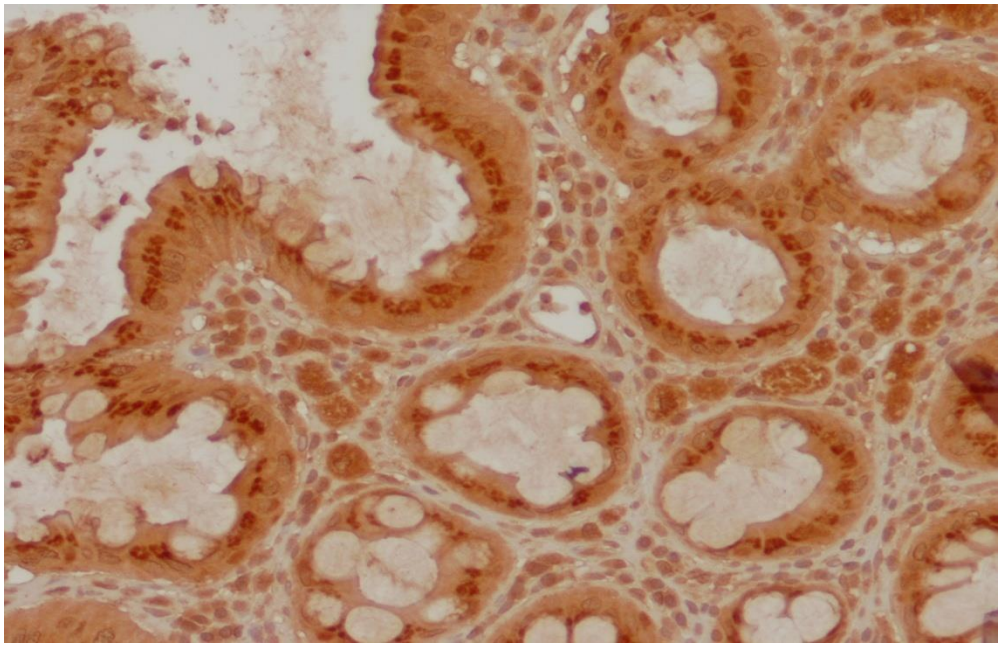


Figure 3.4A: Positive GPR41 nucleus staining in normal mucosa

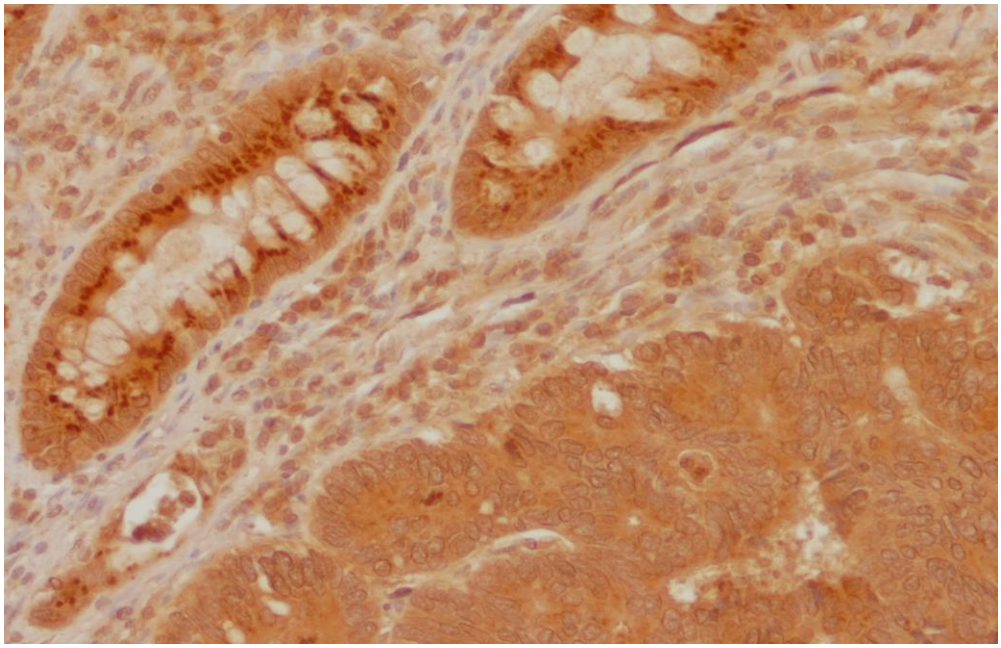


Figure 3.4B: Loss of GPR41 nucleus staining in tumour

3.4 Discussion

Tumours from the caecum, sigmoid colon and rectum were selected to represent the range of tumours occurring in the colon and rectum. 15 cases in total (ie: 5 from caecum, 5 from sigmoid colon and 5 from rectum) were selected to reflect differences in lumen concentration of butyrate from caecum to rectum. In order to minimise compounding factors, all selected cases were moderately differentiated tumours. Only one received pre-operative radiotherapy.

There was no correlation or trend between the low level of positive nucleus staining in tumour tissue and neither evidence of lymphovascular invasion nor the stage of the cancer itself (Table 3.2 & 3.3). Perhaps the sample size was too small to detect significant differences. Neither GPR43 nor GPR41 should be excluded as prognostic markers in colorectal cancer.

The finding of nucleus staining indicated that GPR43 and GPR41 were both present in the nucleus. This is the first report of such findings in the English literature. The roles of nucleus GPR43 and GPR41 are open to speculation. It is possible that both GPR43 and GPR41 are either nucleus membrane receptors, nucleus transport proteins or part of a large, complex protein structure involving in regulator function of transcription of genes. The latter is likely to be true as butyrate is known to induce apoptosis by inhibition of histone deacetylase activity (Sealy & Chalkley 1978). A recent publication by Tang et al. (2011) confirmed findings that GPR43 expression was reduced in colorectal cancer. The significant difference in that the present study involved paired samples of normal and cancer tissue, allowing direct comparison between normal and cancer tissue. Secondly, in the Tang et al. study, the number of cancer tissues was twice the number of normal tissues. The disappointing fact in that Tang and co-workers did not investigate GPR41 in the same tissue samples. Tang and his co-workers became the first to publish their findings, even though the present study was among the first to assess the expression of GPR43 and GPR41 in colorectal cancer. There was no publication in the English language literature at the time of this study.

The cause of the trends of increasing GPR41 and decreasing GPR43 positive nucleus staining towards the rectum is unclear. It has been shown that butyrate level is lowest in the rectum and highest in the caecum. Butyrate is more potent on GPR41 (Le Poul et al. 2003). The affinity of butyrate to GPR43 and GPR41 has not been described. It is possible that the affinity of butyrate to GPR41 may be lower than affinity to GPR43 which would explain the higher number of GPR41 present in the rectum. It is possible that GPR41 and GPR43 have different roles. This observation has not been reported before and thus it is open to speculation.

GPR43 and GPR41 were both present in the cytoplasm and nucleus of normal and tumour colorectal epithelium. The positive staining of GPR43 and GPR41 around the golgi apparatus signified that both receptors were been translated from the regular translating process from mRNA to proteins. This further supports the evidence that GPR43 and GPR41 are true proteins (Figure 3.4B). The degree of positive nucleus staining was significantly more in normal epithelium, indicating a significant down regulation of both GPR43 and GPR41 in cancer. This finding suggested that the loss or reduced expression of GPR43 and GPR41 occurs in cancer. The down regulation or lost expression of GPR43 and GPR41 could lead to reduction in apoptosis and subsequent carcinogenesis and tumour proliferation. The mechanism is still to be defined.

There are limitations in this study. First, a sample size of 15 paired normal and tumour tissues is a potential drawback. It is debatable, however, whether a larger number is required as both GPR43 and GPR41 were consistently shown to be more positively stained in normal epithelium than tumour across the 15 paired samples. Consistency in the results suggested that this sample size was adequate. Selection of cases from the tumour bank was as random as possible. Subsequent retrospective review of clinical details attempted to ensure they were well differentiated tumours. This can be interpreted as selection bias. Preparation and staining of slides, however, was performed by the author and qualified pathologists independent of the study were requested to interpret the slides and validate results. The staining of the slides was performed at a later date after obtaining the clinical details. Second, the interpretation and quantification of positive staining were subjective owing to the experience of the qualified pathologists. There was an issue with interpersonal variation in the interpretation of slides but the average results were chosen for both normal and tumour tissues. Percentage was used as a more direct comparison between the matched pair of normal and tumour of the same subjects. Third, cytoplasmic staining was not included in the results because it was difficult to differentiate between cytoplasmic and surface membrane staining. Furthermore, cytoplasmic staining can also be non-specific background staining, and although it was positive on staining, it was advised it should be omitted from the results.

For the first time GPR43 and GPR41 were shown to be present in human colorectal tumour tissue. Butyrate is one of the short chain fatty acids produced from bacterial fermentation of dietary fiber in the human lower gastrointestinal tract. Epidemiological studies suggest dietary fiber has protective effects in preventing colorectal cancer however the mechanisms behind these protective effects have not been elucidated. The finding of reduced positive nucleus staining of both GPR43 and GPR41 in tumour suggested both a potential role in apoptosis and

that the down regulation of these proteins contributed to the carcinomatosis process. These findings should raise more questions and promote further research into dietary fiber.

3.5 Conclusion

Both GPR43 and GPR41 were present in normal colorectal epithelium and cancer but the nucleus staining of GPR43 and GPR41 was more intense in normal colorectal epithelium than in cancer tissue. The findings suggested that GPR43 and GPR41 expression are down regulated in cancer. It is indirectly confirmed the hypothesis that GPR43 and GPR41 are down-regulated in cancer. Although what causes the loss or reduction in GPR43 and GPR41 expression have not been identified, it is speculated that GPR43 and GPR41 are involved in cell apoptosis and the loss or reduction of these receptors results in the development of colorectal cancer.

Chapter Four: Real-time RT-PCR of GPR43 and GPR41 in Colorectal Tissue

Overview

Real-time RT-PCR of GPR43 and GPR41 in colorectal cancer has not been described. The study aims to determine the mRNA expression of GPR43 and GPR41 and to compare the level of expression between normal and cancer colorectal epithelium. 15 matched pairs of normal and cancer tissue of the same 15 subjects as in chapter 3 were selected. The tissues were stored in RNAlater solution at -80^{o}C. RNA extraction, RNA clean up and real-time RT-PCR were performed. GPR43 and GPR41 expressions were higher in cancer. The finding was contradicting to the earlier findings from chapter 3. The up-regulated of expression of GPR43 and GPR41 in cancer tissue is likely to be a false positive finding, contributed by the mRNA of non cancer cells.

4.1 Introduction

Preventing the development of cancer is better than cure. As the incidence of colorectal cancer is increasing, ongoing colorectal cancer research is vital (Committee 2005). GPR43 and GPR41 have been shown to be expressed in the human lower gastrointestinal tract but not in colorectal cancer (Karaki et al. 2008; Tazoe et al. 2009). Although Tang and co-workers (2011) first reported the presence and expression of GPR43 in human colorectal cancer, GPR41 was not included in their study. Butyrate induces apoptosis and GPR43 and GPR41 are receptors of butyrate. The roles of these receptors in human colorectal epithelium remain to be defined.

It has been demonstrated in chapter 3 that both GPR43 and GPR41 were present in both normal colorectal epithelium and cancer and that both GPR43 and GPR41 were significantly down regulated in cancer. This study employs real-time RT-PCR and aims to assess the mRNA expression of GPR43 and GPR41 and compares it directly across paired samples of normal and cancer tissue from the same subjects. Direct comparison of GPR43 and GPR41 expression in normal and cancer tissue of the same subjects provides a unique insight to biological behaviour in cancer. The hypothesis is that GPR43 and GPR41 expression is down regulated in cancer.

4.2 Methods

4.2.1 Subjects

15 paired normal colorectal mucosa and tumour tissue from 15 subjects with CRC, consisting of 5 caecal, 5 sigmoid colon and 5 rectal cancers. Tissues were labelled from 1 to 30 in pairs. The mucosal tissue was collected within 15 minutes of the bowel being resected. A 2 cm strip of mucosa only was sampled from the bowel and stored in RNAlater solution at -80oC. RNAlater (Qiagen, Melbourne Australia) to reduce RNA degradation by RNAse liberated from dead cells. Normal mucosal tissue was sampled from the most proximal end of the resected colon and the tumour was sampled at the tumour edge.

4.2.2 Essential items

Real-time RT-PCR GPR43 primer assay (Detected transcript NM_005306) and GPR41 primer assay (Detected transcript NM_005304) were purchased from Qiagen, Melbourne, Australia. ACTB primer assay (Detected transcript NM_001101) was purchased from Jomar Bioscience, Adelaide, Australia.

4.2.3 RNA extraction

RNA was extracted using RNeasy Mini Kit (Qiagen, Melbourne, Australia), with modification of the existing protocol to achieve higher RNA yield and improved quality RNA.

Things to do before starting

- 10 µl of β-Mercaptoethanol (β-ME) is added to every 1 ml of Buffer RLT.
- 4 volume of ethanol (96-100%) is added to Buffer RPE as per protocol.
- DNase stock solution is re-constituted as per protocol and aliquot in 60 µl volume.
- 70% and 95% ethanol is prepared from high grade RNA free ethanol (Sigma-Aldrich, Sydney, Australia)

Tissue homogenization

Tissue dissection

- Frozen tissue stored in RNAlater solution was retrieved from -80oC freezer.
- Thawing of the tissue at room temperature.
- Approximately 30 mg of mucosa (both normal colorectal mucosa and tumour) was dissected from the stored tissue sample, using aseptic technique with sterile scalpel and sterile plastic container.
- Mincing of the tissue with scalpel and transfer to 1.5 ml eppendorf tube for disruption and homogenisation.

Tissue disruption and homogenization protocol

- 600 µl of Buffer RLT is added to the 1.5 ml eppendorf tube, rotor-pestle is used to homogenise the tissue. Avoid excess heat production during the process as RNA can be degraded at high temperate generated by friction from the rotating pestle against the eppendorf tube.
- Pipette the lysate into the QIA shreder spin column placed in a 2 ml collection tube and centrifuge for 4 minutes at full speed.
- Supernatant is removed by pipetting and transfer it to a new 1.5 ml eppendorf tube.
- Add 1 volume of 70% ethanol to the clear lysate, mix immediately by gently pipetting and do not centrifuge the lysate and do not discard any precipitates as they are RNA.
- Should proceed immediately to the next crucial step.
- Transfer mixed sample including any precipitate into the RNeasy Mini spin column in a 2 ml collection tube, close the lid and centrifuge for 20 seconds at 10,000 rpm or 8000 x g. Discard the flow-through or collect the flow-through for future DNA and protein extraction. Reuse the collection tube for the next step.
    - If the sample volume exceeds 700 µl, centrifuge the successive aliquots in the same RNeasy Mini spin column, and discard the flow-through. Reuse the collection tube.
- Add 500 µl of Buffer RW1 to the RNeasy Mini spin column, close the lid and wait for 5 minutes on the bench top before centrifuging for 20 sec at 10,000 rpm or 8,000 x g to wash the spin column membrane. Discard the flow-through. Care should be taken to avoid the column being in contact with the flow-through. Reuse the collection tube.

- Add further 500 µl of Buffer RW1 to the RNeasy Mini spin column, close the lip and centrifuge for 20 sec at 10,000 rpm, and discard the flow-through. Reuse the collection tube.
- Add 500 µl of Buffer RPE to the RNeasy Mini spin column, close the lid and centrifuge at 10,000 rpm for 20 sec. Discard the flow-through and reuse the collection tube.
- Add 500 µl of Buffer RPE to the RNeasy Mini spin column, close the lid and centrifuge at 10,000 rpm for 2 minutes. Discard the flow-through and reuse the collection tube.
- 2 minutes of centrifugation would dry the RNeasy Mini spin column, preventing any ethanol being carried over during RNA elution as residual ethanol may interfere with downstream reactions.
- Carefully place the RNeasy Mini spin column in a new 2 ml collection tube and discard the collection tube with flow-through. Close the lid and centrifuge at full speed for 1 minutes to eliminate any possible carryover of Buffer RPE.

RNA elution steps protocol

- Place the RNeasy Mini spin column in a new 1.5 ml (use own autoclaved 1.5 ml eppendorf tubes).
- Add 43 µl of RNase-free water directly onto the spin column membrane to avoid carryover the Buffer RPE left above the spin column membrane, close the lid and centrifuge for 1 minute at 10,000 rpm to elute the RNA from the column.
    - Repeat the previous step with the same amount of RNase-free water.
- Transfer the RNA solution into a new 1.5 ml eppendorf tube (own autoclaved tubes) to avoid DNA contamination during the centrifugation process.

DNase treatment steps

- In solution treatment is more effective than on column treatment in samples with significant DNA contamination.
- 86 µl of RNA solution.
- 20 µl Buffer RDD.
- 5 µl DNase I stock solution.
- Gently mixing with by inverting the tube, then a quick centrifuge (2 seconds).
- Incubate at 37oC for 30 minutes.

- Follow RNA clean up protocol.

DNase treatment protocol

- Before use DNase (Qiagen, Melbourne Australia) stock solution is re-constituted as per protocol.
- 500 µl of RNase-Free Water is injected into the glass vial, using 1 ml syringe and 19FG needle.
- Dissolve of the lyophilosed DNase I is achieved by inverting the vial.
- The stock solution is aliquot in 60 µl volume in a 200 µl eppendorf tube.
- The aliquot DNase solution is stored at -20oC to maintain its effectiveness.
- Thaw at room temperature before use.

4.2.4 RNA clean up protocol

- Add 350 µl of Buffer RLT and mix well.
- Add 250 µl of 95% ethanol and mix well by pipetting. Do not centrifuge, proceed immediately to the next step.
- Transfer the sample in whole or divide aliquot to the RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid and centrifuge for 20 seconds at 10,000 rpm. Discard the flow-through and re-use the collection tube.
    - Care should be taken to avoid the column being in contact with the flow-through.
    - Ensure emptying the collection tube completely.
- Add 500 µl Buffer RW1 to the RNease Mini spin column, close the lid and centrifuge for 20 seconds at 10,000 rpm, to wash the spin column membrane. Discard the flow-through and re-use the collection tube.
- Add 500 µl Buffer RPE to the RNease Mini spin column, close the lid and centrifuge for 20 seconds at 10,000 rpm, to wash the spin column membrane. Discard the flow-through and re-use the collection tube.
- Add 500 µl Buffer RPE to the RNease Mini spin column, close the lid and centrifuge for 2 minutes at 10,000 rpm, to wash the spin column membrane.
- Place the RNeasy Mini spin column in a new 2 ml collection tube and discard the flow-through and the collection tube. Care should be taken to avoid the column being in contact with the flow-through. Centrifuge at full speed for 1 minute to eliminate any possible carryover of Buffer RPE

RNA elution steps protocol

- Place the RNeasy Mini spin column in a new 1.5 ml (use own autoclaved 1.5 ml eppendorf tubes)
- Add 43 µl of RNase-free water directly onto the spin column membrane to avoid carryover the Buffer RPE left above the spin column membrane, close the lid and centrifuge for 1 minute at 10,000 rpm to elute the RNA from the column.
    - Repeat the previous step with the same amount of RNase-free water.
- Transfer the RNA solution into a new 1.5 ml eppendorf tube (own autoclaved tubes) to avoid DNA contamination during the centrifugation process.
- Aliquot of RNA samples for confirmation of clearance of DNA contamination by real-time RT-PCR and quantification by photospectometer
- Label the samples and mix well by pipetting then transfer 2 µl of RNA solution to another 0.5 ml autoclaved eppendorf tube.

Ready for real-time RT-PCR for confirmation of DNA contamination and quantification by photospectometer

All samples should be stored in -80oC freezer

4.2.5 Real-time RT-PCR protocol

Corbett, Rotor-gene RT-PCR machine (Corbett research, Sydney, Australia): 72 wells per run.
Real-time RT-PCR mixture prepared in 96 wells plate.
Each run included 2 no template controls.
400 ng/reaction in 20 µl reaction

400 ng of total RNA
10 µl Master Mix
2 µl of RT-PCR primers mix for GPR43 and GPR41
0.8 µl of RT-PCR primers mix for ACTB
0.6 µl of RT Mix
Volume of ultra-filtered RNA free distilled water to make up 20 µl

Real-time RT-PCR preparation protocol

- Prepare Master mix, RT-PCR primers and RT Mix in 1 or divided eppendorf tubes
- Prepare serial dilution, 1/10, 1/100, 1/500, 1/1000 for GPR43 and GPR41
  1/10, 1/100, 1/1000, 1/10000 for ACTB

1/10	1/100	1/500	1/1000
1 µl + 9 µl	1.5 µl + 13.5 µl	2 µl + 8 µl	1 µl + 9 µl

1/10	1/100	1/1000	1/10000
1 µl + 9 µl	1 µl + 9 µl	1 µl + 9 µl	1 µl + 9 µl

- **Things to avoid contamination of the RT-PCR reactants:**
  - Wear a mask and gloves.
  - Do not cross hands over the open RT-PCR tubes.
  - Pick up the RT-PCR tubes without touching the opening.
  - Place the RT-PCR tubes on the aluminum plate by using a forceps, ensure only pick up the upper part of the tubes.

- Place the aluminum plate on ice, do not use excessive amount of ice to avoid water condensation in the tubes.
- Pipette the different calculated volume of the ultra-filtered RNA free distilled water into the tubes. Replace pipette tip after 8 tubes to avoid residual water remain on the tip.
- Ensure the water is empty into the bottom of the tube.
- Pipette the calculated volume of Master Mix/RT-PCR primers/RT-Mix, replace the pipette tip after 8 tubes to avoid residual solution on the tip.
- Pipette the calculated volume of the total RNA into the designated tube number.
- Mix with solution already in the tube by pipetting 5 times then empty the rest into the tube.
- Ensure no contamination to the covers of the tube when taking them out of the plastic bag.
- Place the cover over the tube without contaminating the mixture of the tubes with your own DNA.
- Label the tube number same number as the tubes are placed on the aluminum plate.
- Mixing the solution by gently flicking and tilting the tubes.

- Place the aluminum plate on ice as waiting to place the tubes into the RT-PCR machine.

4.2.6 Rotor-Gene setting

Profile setting

Hold 1: Hold temperature at 50^{o}
Hold time at 30 minutes
Rotor speed: Normal speed

Hold 2: Hold temperature at 95^{o}
Hold time at 15 minutes
Rotor speed: Normal speed

Cycling: 94^{o} for 15 seconds
55^{o} for 30 seconds
72^{o} for 30 seconds

Melt: Ramp from 72^{o} to 95^{o}
Rising by 1^{o} each step
Wait for 45 seconds on first step then
Wait for 5 seconds for each step afterwards
Acquire to Melt A on FAM/Sybr

Results generated from the Rotor-Gene 6.1 (Corbett research, Sydney, Australia) with the Threshold set as default setting (Auto-find threshold). Each sample was run twice and the mean was calculated.
The final copies/reaction was calculated by dividing the mean copies of interested genes over the mean copies of the housekeeping gene.

4.2.7 Statistics

Paired-sample t-test and Pearman's correlation tests were employed, using SPSS 17.0 (Chicago, IL), p value of < 0.05 was considered to be significant results.

4.3 Results

4.3.1 Subjects

As described in chapter 3, 15 paired-samples from 15 subjects who underwent surgical resection between March 2004 and July 2005 were selected. These were 10 men and 5 women, with a mean age of 71.3 ± 7.46 years (58-87 years). Fifteen tumours were from different sites, five from caecum, five from sigmoid colon and five from rectum. All tumours were well differentiated. They were all early adenocarcinoma, in either stage I or IIA. Lymphovascular invasion (LVI) was reported in 4 subjects. Only one subject (low rectal cancer) received neo-adjuvant chemo-radiotherapy (Table 4.1).

Tumour site	Age	Gender	Pathological grade	LVI	TNM	Stage	Neo-adjuvant RT
Caecum	70	F	MDC	No	T3N0	IIA	No
Caecum	69	M	MDC	No	T3N0	IIA	No
Caecum	87	M	MDC	Yes	T3N0	IIA	No
Caecum	78	F	MDC	No	T3N0	IIA	No
Caecum	72	F	MDC	No	T3N0	IIA	No
Sigmoid	76	M	MDC	Yes	T3N0	IIA	No
Sigmoid	67	M	MDC	Yes	T3N0	IIA	No
Sigmoid	61	M	MDC	No	T3N0	IIA	No
Sigmoid	74	M	MDC	No	T3N0	IIA	No
Sigmoid	77	M	MDC	Yes	T2N0	I	No
Rectal	58	F	MDC	No	T1N0	I	Yes
Rectal	75	M	MDC	No	T1N0	I	No
Rectal	66	F	MDC	No	T3N0	IIA	No
Rectal	64	M	MDC	No	T2N0	I	No
Rectal	76	M	MDC	No	T3N0	IIA	No

Table 4.1: Demographic details and pathological status of 15 subjects

4.3.2 Real-time RT-PCR: Normal mucosa vs. tumour

Similar to Immunohistochemistry staining, both GPR43 and GPR41 expression was detected in normal and tumour colorectal mucosa. The expression level was low in comparison to house keeping gene such as ACTB. GPR43 expression was much greater than GPR41 (Figures 4.1).

- Colorectal normal and tumour mucosa
  - Low mRNA expression

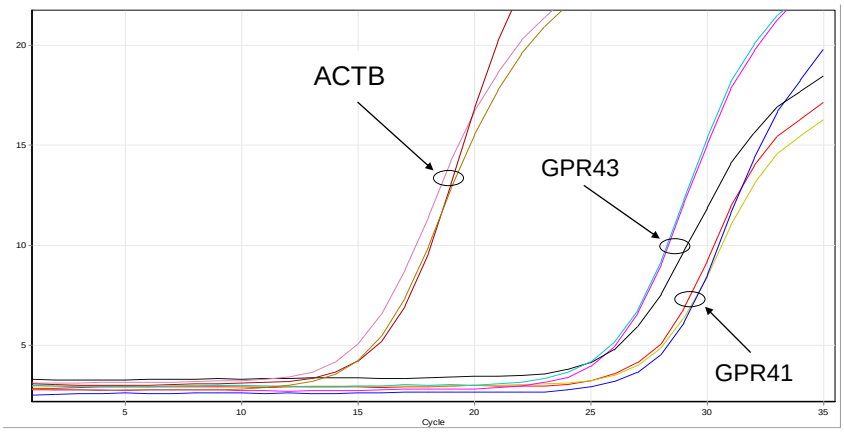


Figure 4.1: Relative expression of GPR43, GPR41 and ACTB in normal mucosa and tumour

The level of expression of the studied genes GPR43 and GPR41 was presented against a common housekeeping gene, ACTB. Results were shown as a relative expression with ACTB as house keeping gene. Due to the complexity of RT-PCR the exact level of gene expression could not be determined.

The expression of GPR43 was highest in normal sigmoid colon mucosa, 15.97 ± 11.63 (mean ± SD) (Table 4.2 and figure 4.2), whereas expression of GPR43 was higher in caecum and rectum in tumour mucosa and highest in the rectum (Table 4.2 and figure 4.3). The changes for GPR43 between normal and tumour was greatest in sigmoid colon lesions, suggesting that sigmoid colon carcinomas are biologically different tumours (Figure 4.4). In addition, the ratio of GPR43/41 was highest in the sigmoid colon for both normal and tumour mucosa (Figure 4.5). The significance of this finding is unknown.

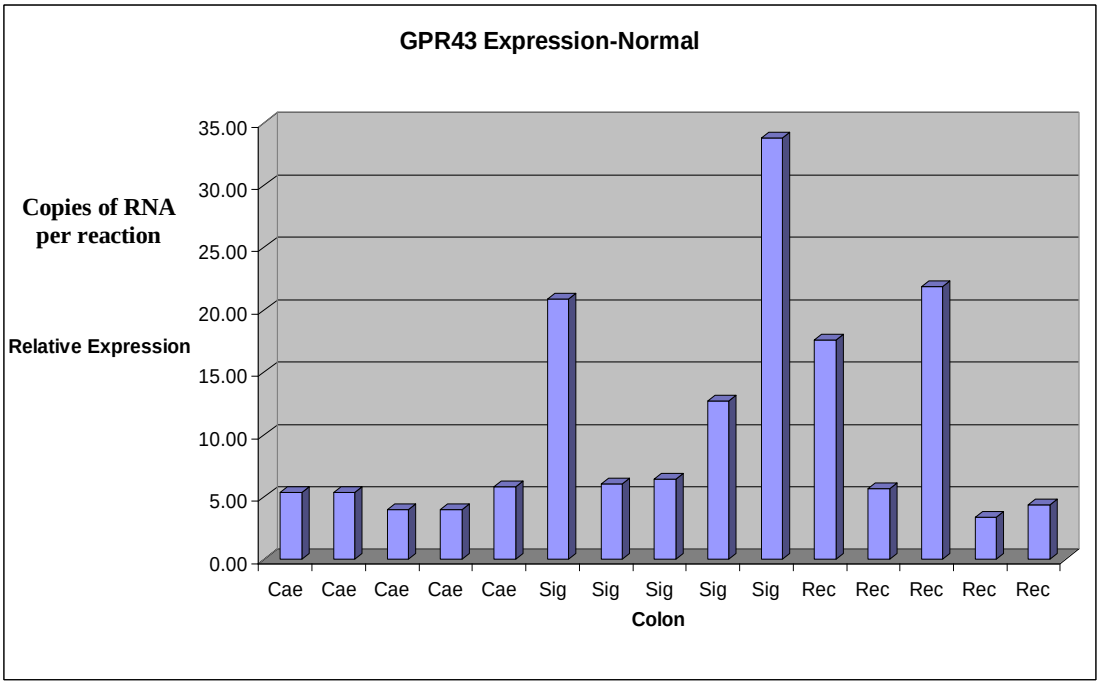


Figure 4.2: Relative expression of GPR43 in normal mucosa

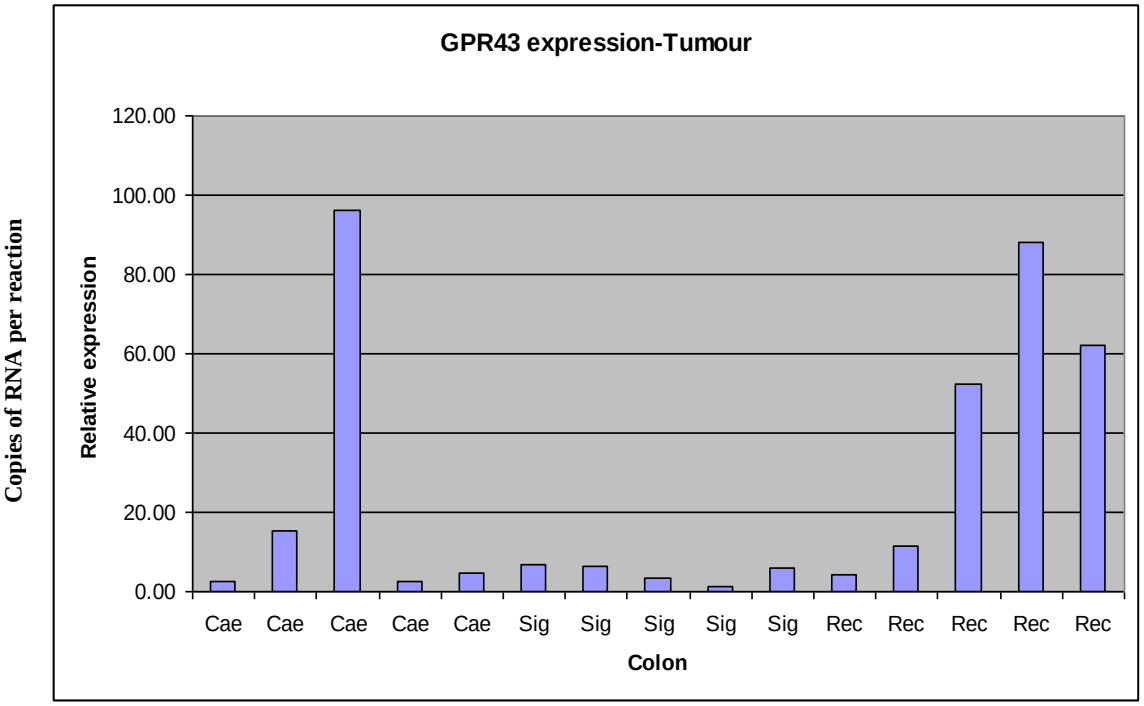


Figure 4.3: Relative expression of GPR43 in tumour

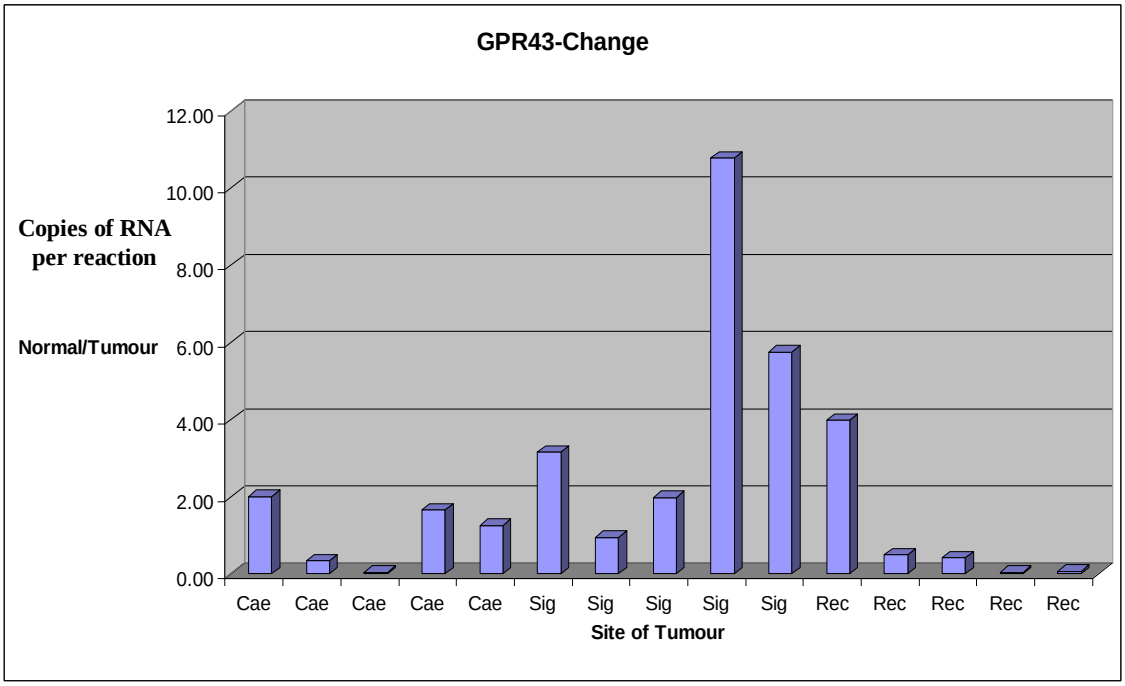


Figure 4.4: The changes between normal mucosa and tumour GPR43 by site of tumour

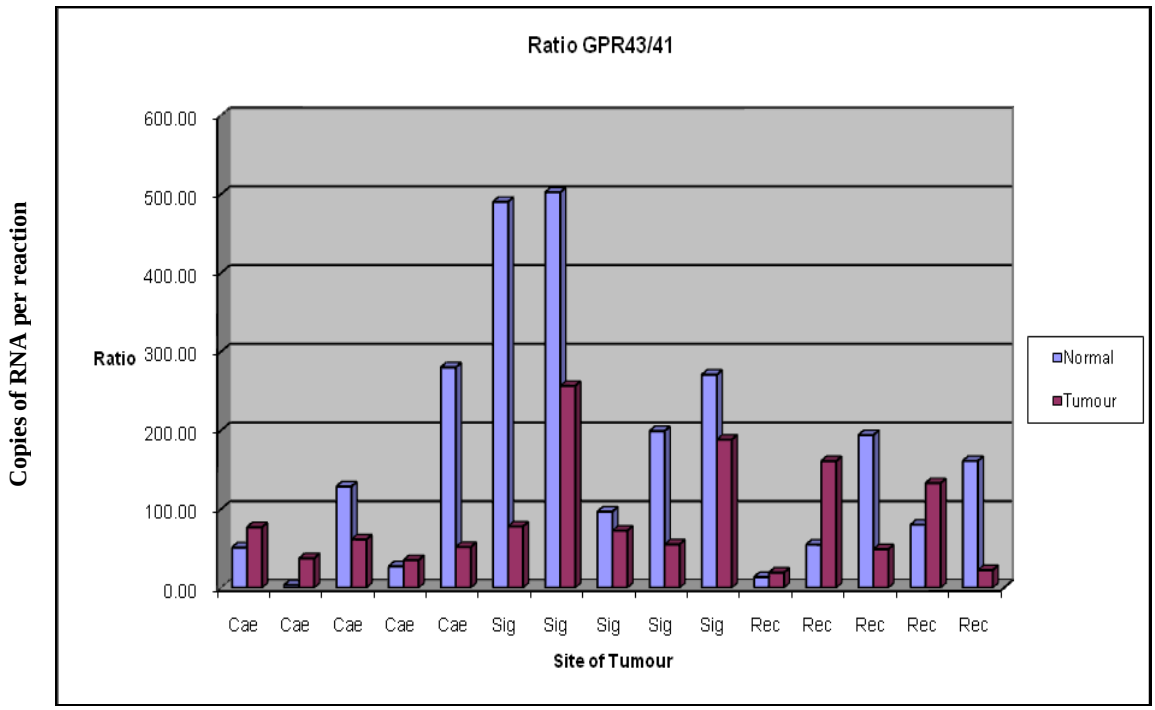


Figure 4.5: The ratio of GPR43/41 by site of tumour

The relative level of GPR41 expression was very much lower than GPR43 in both normal and tumour mucosa, 0.24 ± 0.47, 0.48 ± 0.78, 10.47 ±9.03 and 24.16 ±33.08 respectively (mean ± SD) (Table 4.2 and figures 4.6, 4.7, 4.8). The relative level of GPR43 expression was twice as high in tumour mucosa as normal mucosa (24.16 ± 33.08 vs. 10.47 ± 9.03, mean ± SD), similar finding for GPR41 (0.48 ± 0.78 vs. 0.24 ± 0.47). The differences were not statistically significant ($p > 0.05$) (Table 4.2 and figure 4.6).

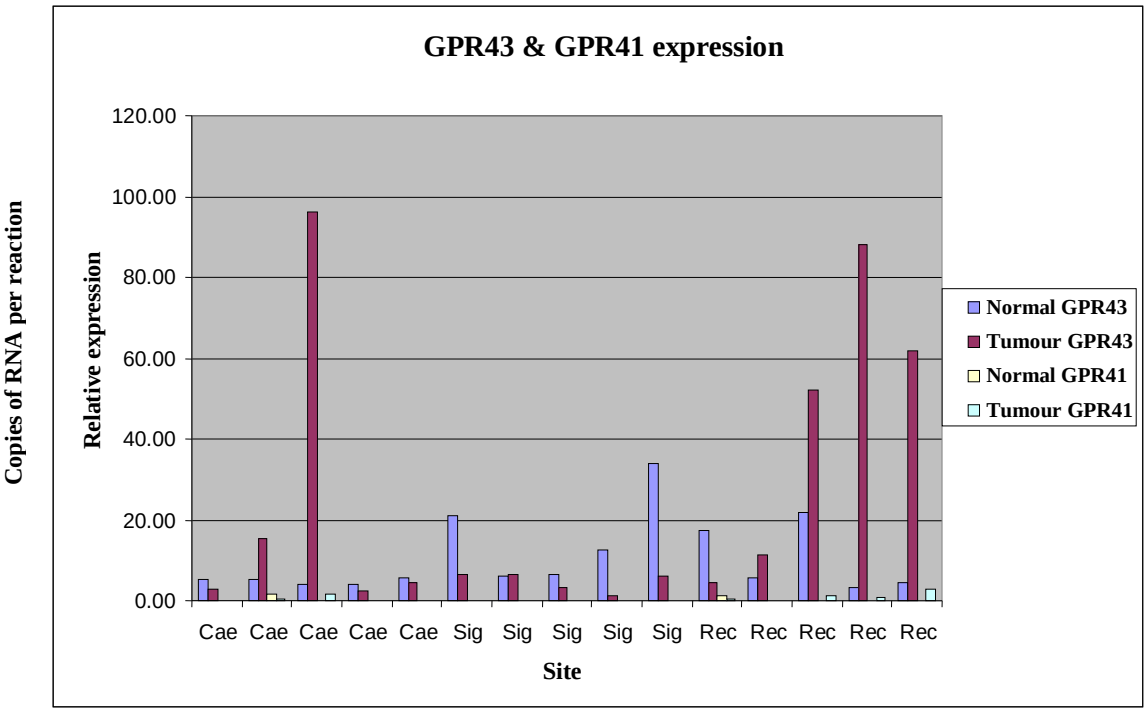


Figure 4.6: Relative expression of GPR43 and GPR41

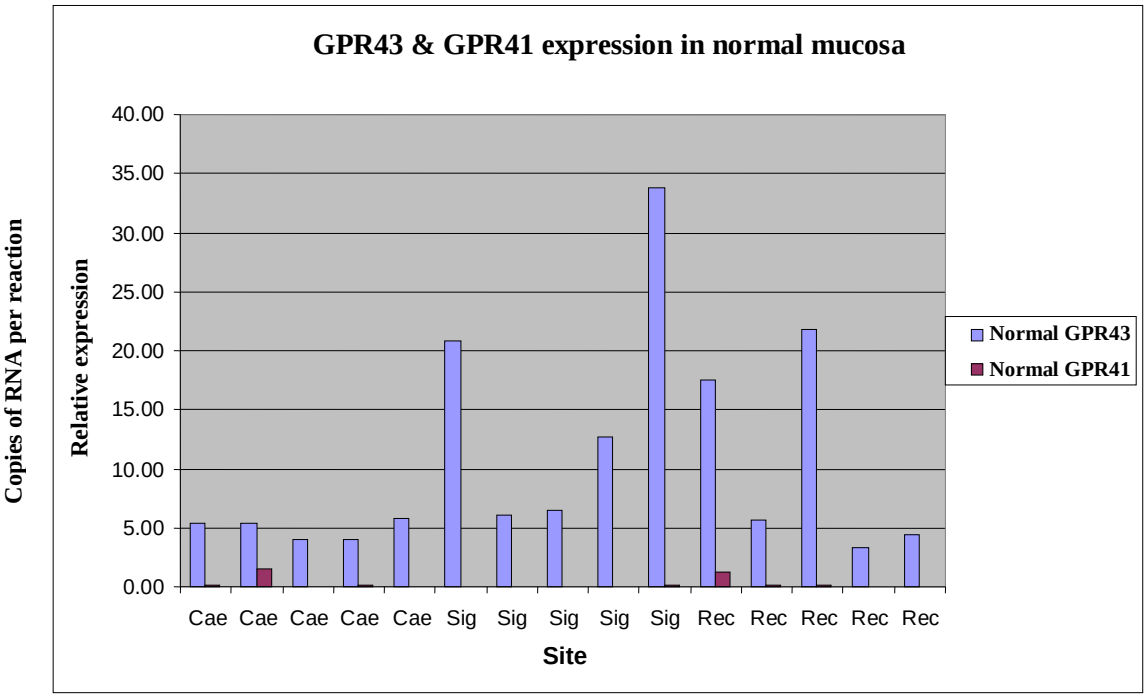


Figure 4.7: GPR43 and GPR41 expression in normal mucosa

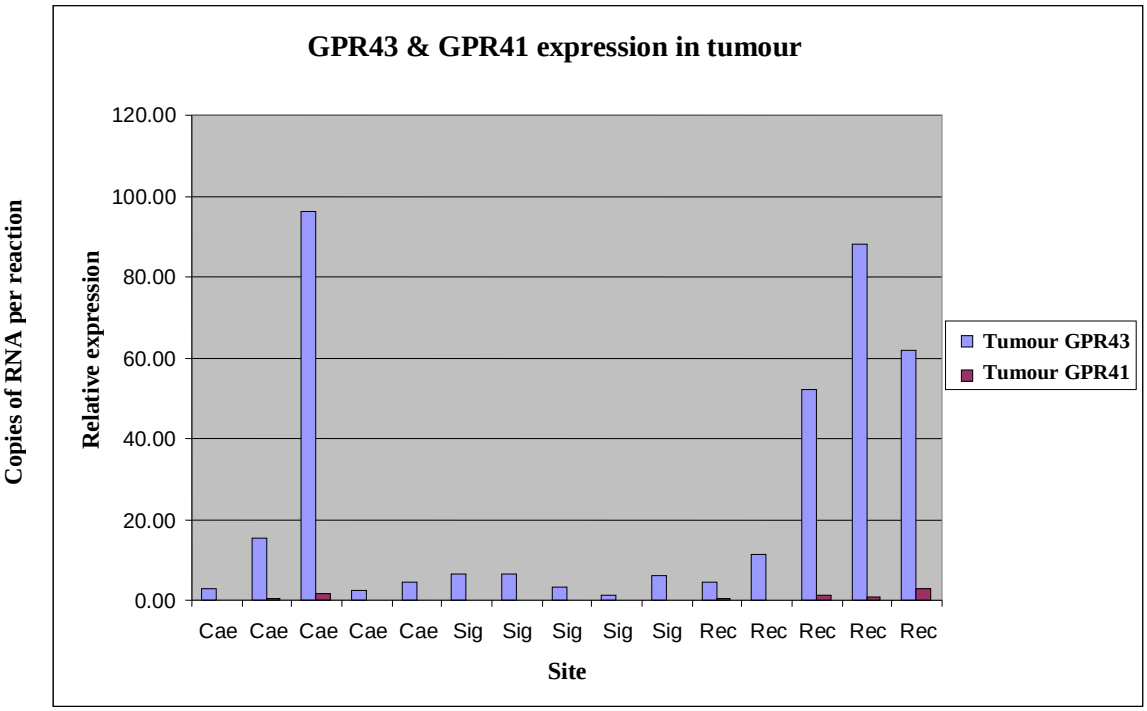


Figure 4.8: GPR43 and GPR41 expression in tumour

Site	Normal GPR43	Mean ± SD	Tumour GPR43	Mean ± SD	Normal GPR41	Mean ± SD	Tumour GPR41	Mean ± SD
Caecum	5.35		2.67		0.10		0.03	
Caecum	5.37		15.43		1.50		0.41	
Caecum	3.94	4.89 ± 0.85	96.13	24.25 ± 40.54	0.03	0.36 ± 0.64	1.56	0.43 ± 0.65
Caecum	3.99		2.41		0.15		0.07	
Caecum	5.78		4.59		0.02		0.09	
Sigmoid	20.88		6.63		0.04		0.08	
Sigmoid	6.04		6.38		0.01		0.02	
Sigmoid	6.44	15.97 ± 11.63	3.26	4.66 ± 2.37	0.07	0.06 ± 0.04	0.04	0.04 ± 0.02
Sigmoid	12.70		1.18		0.06		0.02	
Sigmoid	33.79		5.87		0.12		0.03	
Rectum	17.57		4.41		1.29		0.23	
Rectum	5.65		11.38		0.10		0.07	
Rectum	21.85	10.56 ± 8.53	52.16	43.56 ± 35.16	0.11	0.31 ± 0.55	1.06	0.96 ± 1.08
Rectum	3.35		87.89		0.04		0.66	
Rectum	4.38		61.96		0.03		2.76	
Mean ± SD	10.47 ± 9.03		24.16 ± 33.08		0.24 ± 0.47		0.48 ± 0.78	

Table 4.2: Relative expression of GPR43 and GPR41 in both normal mucosa and tumour

Paired-sample t-test	GPR43	GPR41
Normal vs. Tumour	p = 0.167	p = 0.363
Subset analysis of Normal vs. Tumour		
Caecum	p = 0.351	p = 0.872
Sigmoid colon	p = 0.084	p = 0.378
Rectum	p = 0.133	p = 0.362

Table 4.3: Paired-sample t-test

There were no statistically significant differences between the level of expression of GPR43 and GPR41 in normal mucosa and tumour. There were also no significant differences between different sites of tumour in subset analysis (Table 4.3). There was no correlation between the level of GPR43 & GPR41 expression and LVI (Tables 4.4 to 4.6).

Tumour sites	Normal mucosa	Tumour	LVI
Caecum	5.35	2.66	No
Caecum	5.37	15.43	No
Caecum	3.94	96.13	Yes
Caecum	3.99	2.41	No
Caecum	5.78	4.59	No
Sigmoid	20.88	6.63	Yes
Sigmoid	6.04	6.38	Yes
Sigmoid	6.44	3.26	No
Sigmoid	12.70	1.18	No
Sigmoid	33.78	5.87	Yes
Rectum	17.57	4.41	No
Rectum	5.65	11.38	No
Rectum	21.85	52.16	No
Rectum	3.35	87.89	No
Rectum	4.38	61.96	No

Table 4.4: GPR43 and LVI

Tumour sites	Normal mucosa	Tumour	LVI
Caecum	0.10	0.03	No
Caecum	1.50	0.41	No
Caecum	0.03	1.56	Yes
Caecum	0.15	0.07	No
Caecum	0.02	0.09	No
Sigmoid	0.04	0.08	Yes
Sigmoid	0.01	0.02	Yes
Sigmoid	0.07	0.04	No
Sigmoid	0.06	0.02	No
Sigmoid	0.12	0.03	Yes
Rectum	1.29	0.23	No
Rectum	0.10	0.07	No
Rectum	0.11	1.06	No
Rectum	0.04	0.66	No
Rectum	0.03	2.76	No

Table 4.5: GPR41 and LVI

Pearman's correlation test	GPR43	GPR41
LVI	Correlation Coef of 0.04	Correlation Coef of 0.34
	p = 0.89	p = 0.21
Subset analysis		
Caecum	0.612	0.612
	p = 0.27	p = 0.27
Sigmoid colon	0.408	0.667
	p = 0.5	p = 0.22
Rectum	No correlation	No correlation

Table 4.6: The correlation between GPR43, GPR41 and LVI

4.4 Discussion

Real-time RT-PCR of colorectal tumour tissue was not previously described in English language literature. Perhaps this is because of technical difficulties. The difficulties encountered and remedies found were discussed in chapter 2. In this study, the real-time RT-PCR runs were successful and allowed a direct comparison between normal and tumour tissue.

Overall the level of expression for both GPR43 and GPR41 was very low when compared to ACTB housekeeping gene. GPR43 mean expression was 40 and 50 fold more than GPR41 in normal and tumour tissue respectively. Both GPR43 and GPR41 mean expression were double in tumour tissue. This finding contradicted results from immunohistochemistry staining, where more positive nucleus staining was evident in normal colorectal mucosa. The increase in expression of mRNA did not translate into higher protein levels as seen in immunohistochemistry staining. This indicated that possible mutations in the GPR43 and GPR41 DNA sequences result in non-translated mRNA.

Another reason for higher levels of mRNA expression might be that cells other than colorectal epithelial cells were included in the tissue from which RNA was extracted. Immune cells are known to highly express GPR43 and GPR41 (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003; Senga et al. 2003; Xiong et al. 2004). Previous immunohistochemistry study (chapter 3) also indicated that cells other than colorectal epithelium and cancer cells stained positive. Perhaps this was the main cause for the non-significant differences.

GPR43 expression consistently showed higher compared to GPR41 in both normal mucosal and tumour tissue. GPR43 and GPR41 do not share the same level of expression. GPR43 expression is higher in haematopoietic tissues such as spleen and bone marrow whereas GPR41 is widely expressed in many tissues, having the highest level of mRNA expression in adipocytes (Brown et al. 2003; Le Poul et al. 2003; Nilsson et al. 2003; Senga et al. 2003; Xiong et al. 2004) . This study is the first to describe such findings in human colorectal mucosa and tumour, and thus no reference can be used to compare our findings. Colorectal mucosa does contain a significant amount of immune cells in the submucosa as a natural immune barrier in the human lower gastrointestinal tract. It is possible that this is the primary limitation of this study and RNA from immune cells in the submucosa was included in the extracted RNA solution.

It is possible to select out colorectal epithelium and tumour tissues and extract their RNA. A Laser-capture microdissection device would be helpful in selecting out the interest cells group, although for low expression genes this would require a large volume of tissue to extract RNA. Another disadvantage of the laser-capture microdissection device is that it cannot be used in fresh tissue. Tissue is required to

be fixed by alcohol-based precipitation techniques and mounted on a slide. Despite the existence of techniques to retrieve RNA following laser-capture microdissection, the concern would be the denaturing of RNA in fixed dry tissue. Perhaps a laser-capture microdissection could more precisely target the tissue but the amount of tissue required to conduct the present study would render the study impractical because the gene expression was so low. Future research with more precisely targeted tissue and a significantly larger budget would be optimal.

GPR43 expression was highest in normal sigmoid colon tissue and least in sigmoid colon tumour tissue. Histologically and biologically, there is only 1 type of adenocarcinoma arising in the colorectal mucosa. At the molecular level, however, all tumours behave differently because all acquire different genetic mutations. It is believed that cancer arising from sigmoid colon is not a different entity to cancer arising from the caecum or rectum. Again, the quantity of tested tissue was too small to reach a general conclusion. 15 was a too small sample to reach any statistically significant conclusions and comparison of subgroup analysis was again too small a number to have any statistical validity. I did think that subgroup analysis was unnecessary as, given the small number in each subgroup, such analysis would be inconclusive.

The incidence of colorectal cancer is increasing and molecular biological markers are becoming increasing popular. An example is the k-ras oncogene. The presence of the wild type k-ras oncology gene in tumour tissue signifies better responses from Avastating chemotherapy agent but not necessarily better long term outcomes (Jain et al. 2006). While indicating high risk of metastatic disease, lymphovascular invasion serves as a poor prognostic marker because tumours with metastatic nodal disease do not necessary show evidence of lymphovascular invasion**.** There was no significant association between the level of GPR43 and GPR41 expression and the prognostic marker for lymphovascular invasion. This was consistent with findings from immunohistochemistry staining in chapter 3. GPR43 and GPR41 are unlikely candidates as molecular prognostic markers but cannot be completely excluded because of the small sample size used in this study.

Recent publications by Karaki et al. (2008) and Tazoe et al. (2009)support our findings that human colorectal tissue expressed GPR43 and GPR41 . These papers show results from one human subject only. They claim to be the first group to publish their results, although I had achieved similar results in the same year but had not yet published. The present study is the first to show that GPR43 and GPR41 also express in human colorectal cancer in 15 human subjects.

4.5 Conclusion

GPR43 and GPR41 expression was up regulated in colorectal cancer. This result is in sharp contrast to my earlier immunohistochemistry study. It is believed that this up regulation was a false positive finding because of mRNA contributed by non cancerous cells.

Chapter Five: Cell Lines HT-29 and SW620

Overview

Human colorectal cancer cell lines allow in-vitro research into the biological behaviour of cancer. HT-29 and SW620 are the two well known colonic cancer cell lines, which have been widely researched. HT-29 and SW620 cell lines were purchased, resuscitated and cultured. The cell lines were cultured until they became confluent. Butyrate at concentrations of 5, 10 and 15 mM were added and incubated at 2, 4, 6 and 12 hours. Zero hour of incubation was set as the control. Cell lines were harvested, RNA extraction, RNA clean up and real-time RT-PCR were performed. Both HT-29 and SW620 expressed GPR43 and GPR41, the level GPR43 expression was significantly higher than GPR41. Butyrate significantly down regulated the expression of both GPR43 and GPR41 in HT-29. These findings were not observed in SW620. The different biological properties of cancer cell lines may account for the differences.

5.1 Introduction

Human cell lines including normal and cancer cell lines provide a great opportunity to study cell function as investigating human cells in vivo at the molecular biology level is impractical. Cancer cell lines in general are immortal and their biological DNA markers do not alter despite multiple cell divisions. Developing a cancer cell line is technically challenging and time consuming, not to mention enormously expensive. There are many commercially available cancer cell lines including colonic cancer cell lines. The advantage of commercially available colonic cell lines is that experiments can be repeated by different research groups to validate others' results. There is a significant amount of research and many publications on the HT-29 cell line at the molecular biology level. Butyrate is found to induce $p21^{Waf1/Cip1}$ expression in inhibiting cell proliferation on the HT-29 cell line. Working with HT-29 would contribute more knowledge and provide a framework within which to continue research (Coradini et al. 2000).

HT-29 was isolated from a primary colonic tumour in a 44 year old Caucasian female in 1964 (Fogh 1975). The primary tumour was a well differentiated adenocarcinoma and it has 46 chromosomes. HT-29 does excrete Carcinoembryonic antigen (CEA). SW620 was developed by A Leibovitz (1976). It was isolated from metastatic lymph node. The primary was poor adenocarcinoma Dukes' C colonic cancer, from a 51 year old Caucasian male in 1975. It has 50

chromosomes but it can be ranged from 45 to 53 chromosomes. Small quantity of CEA is excreted by SW620. There is no report in the literature regarding whether HT-29 or SW620 express GPR43 and GPR41.

From chapter 3, GPR43 and GPR41 were nucleus receptors and that they were stained more intensely in normal human colorectal epithelium than cancer cells. Results from chapter 4, however, do not support earlier findings of chapter 3. The discrepancy in the results has been discussed in chapter 4. This study aims to assess the effects of butyrate on the expression of GPR43 and GPR41 in the colonic cancer cell line, namely HT-29 and SW620. The hypothesis is that butyrate induces down regulation of GPR43 and GPR41 expression in HT-29 and SW620.

5.2 Methods

5.2.1 Cell Lines

Commercially available CRC HT-29 and SW620 cell lines were purchased from Sigma-Aldrich (Sydney, Australia). The cell lines were resuscitated from liquid nitrogen storage as per company protocol.

Resuscitation of cell lines protocol

- Protective laboratory coat, full protective face mask and gloves.
- Cell line removes from the liquid nitrogen tank.
- Leave the ampoule at room temperature for approximately 1 minute and transfer to a 37^{o}C waterbath for 1 minute or until fully thawed. Do not totally immerse the ampoule in the waterbath as this may increase the risk of contamination. Thaw quickly to minimize any damage to the cell membrane.
- Spray the ampoule with 70% alcohol and place it on the bench of the fume hood.
- Pipette the whole contain of the ampoule into the T25 culture flask with 10 ml of medium (McCoy's 5A medium with 2 mM L-glutamine, 10% FBS (Fetal Bovine Serum) and 2% of Streptomycin for HT-29, Leibovitz's medium with 2 mM L-glutamine, 10% FBS and 2% of Streptomycin for SW620).
- Incubate at 37^{o}C temperature and 5% CO_2 level.

Cell culturing

- Cells growth was assess every day under light microscope.
- Culture medium was changed every 72 hours to ensure proliferation of cells.
- Cell growth curve for individual cell line was constructed.

Subculturing protocol

- Removal and discard of culture medium.
- Rinse the cell layer with PBS (Phosphate-buffered Saline).
- Add 0.25% trypsin and 0.03% EDTA, and incubate the flask for 5 minutes at 37^{o}C. The volume of trypsin-EDTA solution varies with different volume flasks.
- Detachment of the cells is observed under light microscope.

- Same volume of culture medium is added to neutralise the effects of tripsin.
- Pipette and transfer the contents of the flask into a 15 ml tube.
- Centrifuge at 1200 rpm for 5 minutes, at 26^{o}C to maintain cell survival.
- The cell pellet is preserved and the supernatant is pipetted and discarded.
- 5 ml of fresh culture medium is added into the tube and gently pipetting up and down to dispense the cell pellet. Avoid excessive pipetting air bubbles into the tube.
- The dispensed cells then transfer into a larger or multiple culture flasks.
- Flasks are kept in cell culture incubator set at 37^{o}C and 5% CO_2.

5.2.2 Butyrate incubation

Cell lines were cultured in a 6-well disc. When cells were well confluent in the well, culture medium was removed and new culture medium with butyrate at known concentration of 5, 10 and 15 mM was added and incubated at zero, 2, 4, 6 and 12 hours duration in a cell culture incubator set at 37^{o}C and 5% CO_2. Cell lines were harvested at the end of incubation time. The adequate volume of cells was determined from prior knowledge of RNA extraction.

Cell lines harvesting protocol

- Rinse the cell layer with PBS (Phosphate-buffered Saline).
- Add 0.25% trypsin and 0.03% EDTA, and incubate the flask for 5 minutes at 37^{o}C. The volume of trypsin-EDTA solution varies with different volume flasks.
- Same volume of culture medium is added to neutralise the effects of tripsin.
- Pipette and transfer the content of the flask into a 15 ml tube.
- Centrifuge at 1200 rpm for 5 minutes, at 26^{o}C to maintain cell survival.
- The cell pellet is preserved and the supernatant is pipetted and discarded.

5.2.3 RNA extraction

RNA was extracted using RNeasy Mini Kit (Qiagen, Melbourne, Australia). Modification of the existing manufacturer protocol was made to achieve higher RNA yield and improved quality RNA. The protocols are similar to chapter 4.

Things to do before starting

- 10 µl of β-Mercaptoethanol (β-ME) is added to every 1 ml of Buffer RLT.
- 4 volume of ethanol (96-100%) is added to Buffer RPE as per protocol.
- DNase stock solution is re-constituted as per protocol and aliquot in 60 µl volume.
- 70% and 95% ethanol is prepared from high grade RNA free ethanol (Sigma-Aldrich, Sydney, Australia).

Cell pellets homogenization protocol

- 600 µl of Buffer RLT to cell line pellets in the15 ml tube.
- Use 3 ml syringe with 19 FG needle to aspirate and do 20 passes.
- Transfer clear lysate to 1.5 ml own autoclaved eppendorf tube and centrifuge for 4 minutes at full speed (The remaining steps are similar to tissue RNA extraction, as described in chapter 4).
- Supernatant is removed by pipetting and transfer it to a new 1.5 ml eppendorf tube.
- Add 1 volume of 70% ethanol to the clear lysate, mix immediately by gently pipetting and do not centrifuge the lysate and do not discard any precipitates as they are RNA.
- Proceed immediately to the next crucial step.
- Transfer mixed sample including any precipitate into the RNeasy Mini spin column in a 2 ml collection tube, close the lid and centrifuge for 20 seconds at 10,000 rpm or 8000 x g. Discard the flow-through or collect the flow-through for future DNA and protein extraction. Reuse the collection tube for the next step.
    - If the sample volume exceeds 700 µl, centrifuge the successive aliquots in the same RNeasy Mini spin column, and discard the flow-through. Reuse the collection tube.
- Add 500 µl of Buffer RW1 to the RNeasy Mini spin column, close the lid and wait for 5 minutes on the bench top before centrifuging for 20 sec at 10,000 rpm or 8,000 x g to wash the spin column membrane. Discard the flow-through. Care should be taken to avoid the column being in contact with the flow-through. Reuse the collection tube.
- Add further 500 µl of Buffer RW1 to the RNeasy Mini spin column, close the lip and centrifuge for 20 sec at 10,000 rpm, and discard the flow-through. Reuse the collection tube.
- Add 500 µl of Buffer RPE to the RNeasy Mini spin column, close the lid and centrifuge at 10,000 rpm for 20 sec. Discard the flow-through and reuse the collection tube.
- Add 500 µl of Buffer RPE to the RNeasy Mini spin column, close the lid and centrifuge at 10,000 rpm for 2 minutes. Discard the flow-through and reuse the collection tube.
- 2 minutes of centrifugation would dry the RNeasy Mini spin column, preventing any ethanol being carried over during RNA elution as residual ethanol may interfere with downstream reactions.

- Carefully place the RNeasy Mini spin column in a new 2 ml collection tube and discard the collection tube with flow-through. Close the lid and centrifuge at full speed for 1 minutes to eliminate any possible carryover of Buffer RPE.

RNA elution steps protocol

- Place the RNeasy Mini spin column in a new 1.5 ml (use own autoclaved 1.5 ml eppendorf tubes)
- Add 43 µl of RNase-free water directly onto the spin column membrane to avoid carryover the Buffer RPE left above the spin column membrane, close the lid and centrifuge for 1 minute at 10,000 rpm to elute the RNA from the column.
    - Repeat the previous step with the same amount of RNase-free water.
- Transfer the RNA solution into a new 1.5 ml eppendorf tube (own autoclaved tubes) to avoid DNA contamination during the centrifugation process.

DNase treatment steps

- In solution treatment is more effective than on column treatment in samples with significant DNA contamination.
- 86 µl of RNA solution
- 20 µl Buffer RDD
- 5 µl DNase I stock solution
- Gently mixing with by inverting the tube, then a quick centrifuge (2 seconds).
- Incubate at 37oC for 30 minutes.
- Follow RNA clean up protocol.

DNase treatment protocol

- Before use DNase (Qiagen, Melbourne Australia) stock solution is re-constituted as per protocol.
- 500 µl of RNase-Free Water is injected into the glass vial, using 1 ml syringe and 19FG needle.
- Dissolve the lyophilised DNase I is achieved by inverting the vial.
- The stock solution is aliquot in 60 µl volume in a 200 µl eppendorf tube.
- The aliquot DNase solution is stored at -20oC to maintain its effectiveness.
- Thaw at room temperature before use.

5.2.4 RNA clean up protocol

- Add 350 µl of Buffer RLT and mix well
- Add 250 µl of 95% ethanol and mix well by pipetting. Do not centrifuge, proceed immediately to the next step.
- Transfer the sample in whole or divide aliquot to the RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid and centrifuge for 20 seconds at 10,000 rpm. Discard the flow-through and re-use the collection tube.
    - Care should be taken to avoid the column being in contact with the flow-through.
    - Ensure emptying the collection tube completely
- Add 500 µl Buffer RW1 to the RNease Mini spin column, close the lid and centrifuge for 20 seconds at 10,000 rpm, to wash the spin column membrane. Discard the flow-through and re-use the collection tube.
- Add 500 µl Buffer RPE to the RNease Mini spin column, close the lid and centrifuge for 20 seconds at 10,000 rpm, to wash the spin column membrane. Discard the flow-through and re-use the collection tube.
- Add 500 µl Buffer RPE to the RNease Mini spin column, close the lid and centrifuge for 2 minutes at 10,000 rpm, to wash the spin column membrane.
- Place the RNeasy Mini spin column in a new 2 ml collection tube and discard the flow-through and the collection tube. Care should be taken to avoid the column being in contact with the flow-through. Centrifuge at full speed for 1 minute to eliminate any possible carryover of Buffer RPE.

RNA elution steps

- Place the RNeasy Mini spin column in a new 1.5 ml (use own autoclaved 1.5 ml eppendorf tubes)
- Add 43 µl of RNase-free water directly onto the spin column membrane to avoid carryover the Buffer RPE left above the spin column membrane, close the lid and centrifuge for 1 minute at 10,000 rpm to elute the RNA from the column.
    - Repeat the previous step with the same amount of RNase-free water.
- Transfer the RNA solution into a new 1.5 ml eppendorf tube (own autoclaved tubes) to avoid DNA contamination during the centrifugation process.
- Aliquot of RNA samples for confirmation of clearance of DNA contamination by real-time RT-PCR and quantification by photospectometer

- Label the samples and mix well by pipetting then transfer 2 µl of RNA solution to another 0.5 ml autoclaved eppendorf tube.

Ready for real-time RT-PCR for confirmation of DNA contamination and quantification by photospectometer

All samples were stored in -80oC freezer

5.2.5 Real-time RT-PCR

Real-time RT-PCR mixture prepared in 96 wells plate
6 wells of the same duration of incubation in one run
Each run included 2 no template controls
400 ng/reaction in 20 µl reaction

Essential items

Real-time RT-PCR GPR43 primer assay (Detected transcript NM_005306) and GPR41 primer assay (Detected transcript NM_005304) were purchased from Qiagen, Melbourne, Australia. ACTB primer assay (Detected transcript NM_001101) was purchased from Jomar Bioscience, Adelaide, Australia.

Real-time RT-PCR protocol

Corbett, Rotor-gene RT-PCR machine (Corbett research, Sydney, Australia): 72 wells per run
20 µl reaction consist of 400 ng of total RNA, Master Mix, RT-PCR primers, RT Mix and ultra-filtered RNA free distilled water

400 ng of total RNA
10 µl Master Mix
2 µl of RT-PCR primers mix for GPR43 and GPR41
0.8 µl of RT-PCR primers mix for ACTB
0.6 µl of RT Mix
Volume of ultra-filtered RNA free distilled water to make up 20 µl

Real-time RT-PCR preparation protocol

- Prepare Master mix, RT-PCR primers and RT Mix in 1 or divided eppendorf tubes
- Prepare serial dilution, 1/10, 1/100, 1/500, 1/1000 for GPR43 and GPR41
  1/10, 1/100, 1/1000, 1/10000 for ACTB

1/10	1/100	1/500	1/1000
1 µl + 9 µl	1.5 µl + 13.5 µl	2 µl + 8 µl	1 µl + 9 µl

1/10	1/100	1/1000	1/10000
1 µl + 9 µl	1 µl + 9 µl	1 µl + 9 µl	1 µl + 9 µl

- **Things to avoid contamination of the RT-PCR reactants:**
  - Wearing a mask and gloves.
  - Do not cross hands over the open RT-PCR tubes.
  - Pick up the RT-PCR tubes without touching the opening.
  - Place the RT-PCR tubes on the aluminium plate by using a forceps, ensure only pick up the upper part of the tubes.

- Place the aluminium plate on ice, do not use excessive amount of ice to avoid water condensation in the tubes.
- Pipette the different calculated volume of the ultra-filtered RNA free distilled water into the tubes. Replace pipette tip after 8 tubes to avoid residual water remain on the tip.
- Ensure the water is empty into the bottom of the tube.
- Pipette the calculated volume of Master Mix/RT-PCR primers/RT-Mix, replace the pipette tip after 8 tubes to avoid residual solution on the tip.
- Pipette the calculated volume of the total RNA into the designated tube number.
- Mix with solution already in the tube by pipetting 5 times then empty the rest into the tube.
- Ensure no contamination to the covers of the tube when taking them out of the plastic bag.
- Place the cover over the tube without contaminating the mixture of the tubes with your own DNA.
- Label the tube number same number as the tubes are placed on the aluminium plate.
- Mixing the solution by gently flicking and tilting the tubes.
- Place the aluminium plate on ice as waiting to place the tubes into the RT-PCR machine.

Rotor-Gene Setting

Profile setting

Hold 1: Hold temperature at 50°
Hold time at 30 minutes
Rotor speed: Normal speed

Hold 2: Hold temperature at 95°
Hold time at 15 minutes
Rotor speed: Normal speed

Cycling: 94° for 15 seconds
55° for 30 seconds
72° for 30 seconds

Melt: Ramp from 72° to 95°
Rising by 1° each step
Wait for 45 seconds on first step then
Wait for 5 seconds for each step afterwards
Acquire to Melt A on FAM/Sybr

Results generated from the Rotor-Gene 6.1 (Corbett research, Sydney Australia) with the Threshold set at default setting (Auto-find threshold). Each sample was run 6 times and the mean was calculated.
The final copies/reaction was calculated by dividing the mean copies of interested genes over the mean copies of the housekeeping gene corresponding to the same duration of incubation and 5, 10, 15mM of butyrate.

5.2.6 Statistics

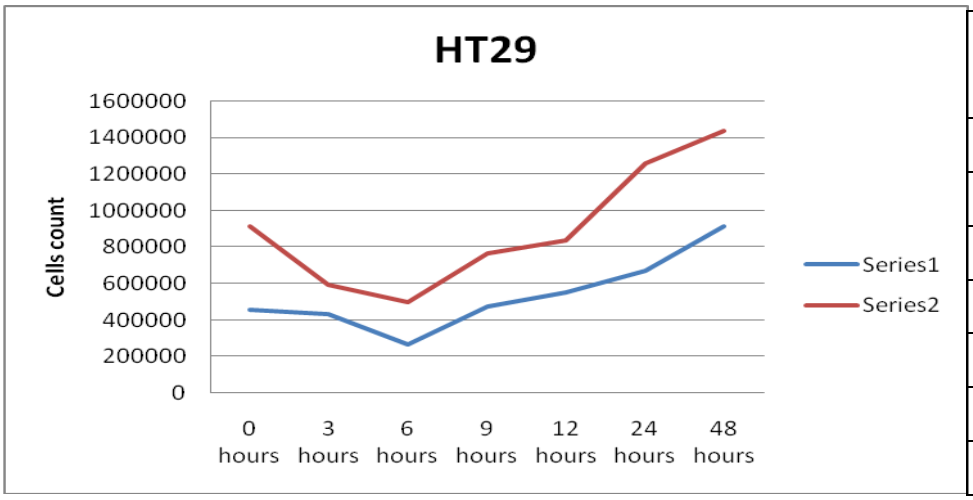
Paired-sample t-test was employed, using SPSS 17.0 (Chicago, IL), p value of < 0.05 was considered to be significant results.

5.3 Results

5.3.1 Growth curve

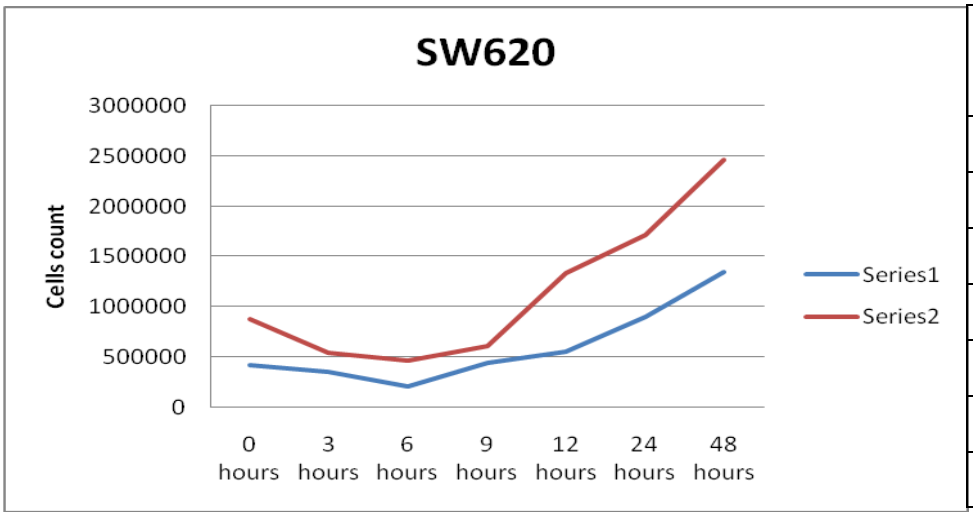
The dynamic of cell growth is important. Observation of cell growth contributes to the understanding of the biological behaviour of cell lines. Doubling time was established by culturing the cells in the appropriate medium and a manual cell count performed to estimate the doubling time.

Doubling times were 48 and 24 hours for HT-29 and SW620 respectively. These doubling times are consistent with the literature, indicating that the culturing technique was optimal for cell growth in our laboratory (Figures 5.1 & 5.2).



Time	Cells count	
	Series 1	Series 2
0 hours	458000	916000
3 hours	430000	593333
6 hours	266666	496666
9 hours	473333	764320
12 hours	553333	833333
24 hours	670000	1256667
48 hours	913333	1436667

Figure 5.1: HT-29 cell line growth curve



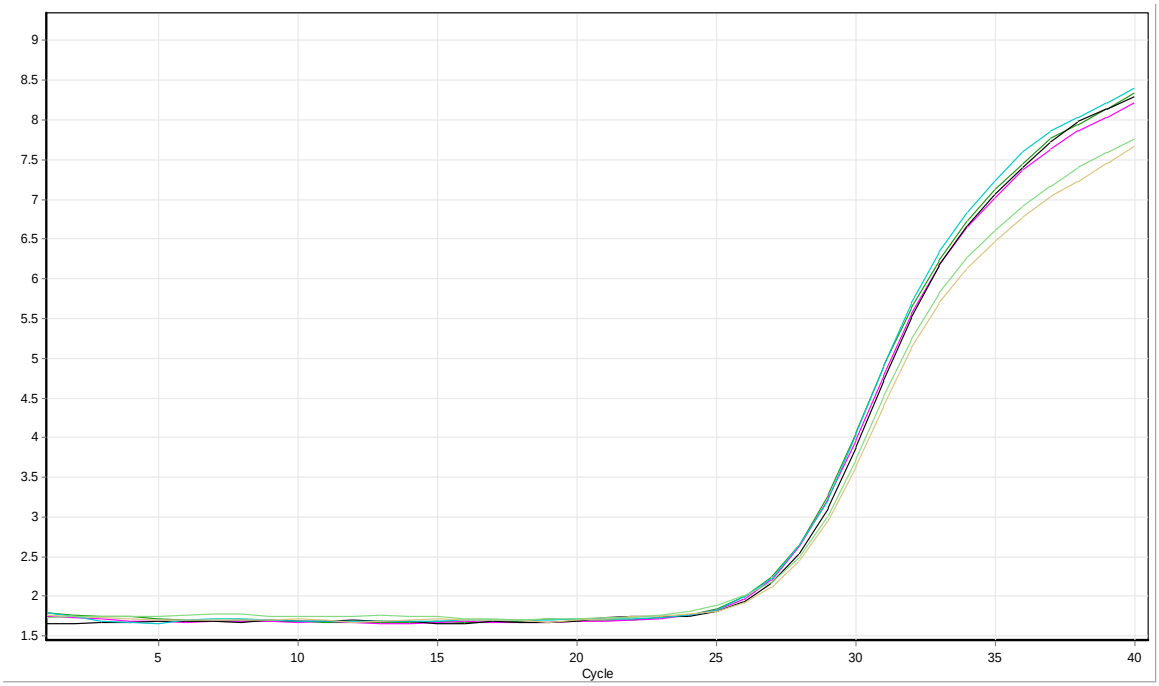
Time	Cells count	
	Series 1	Series 2
0 hours	425200	882600
3 hours	359610	548390
6 hours	214820	469782
9 hours	451070	614455
12 hours	558058	1341580
24 hours	904260	1720936
48 hours	1346020	2463890

Figure 5.2: SW620 cell line growth curve

5.3.2 Real-time RT-PCR of cell lines without butyrate

HT-29

A



B

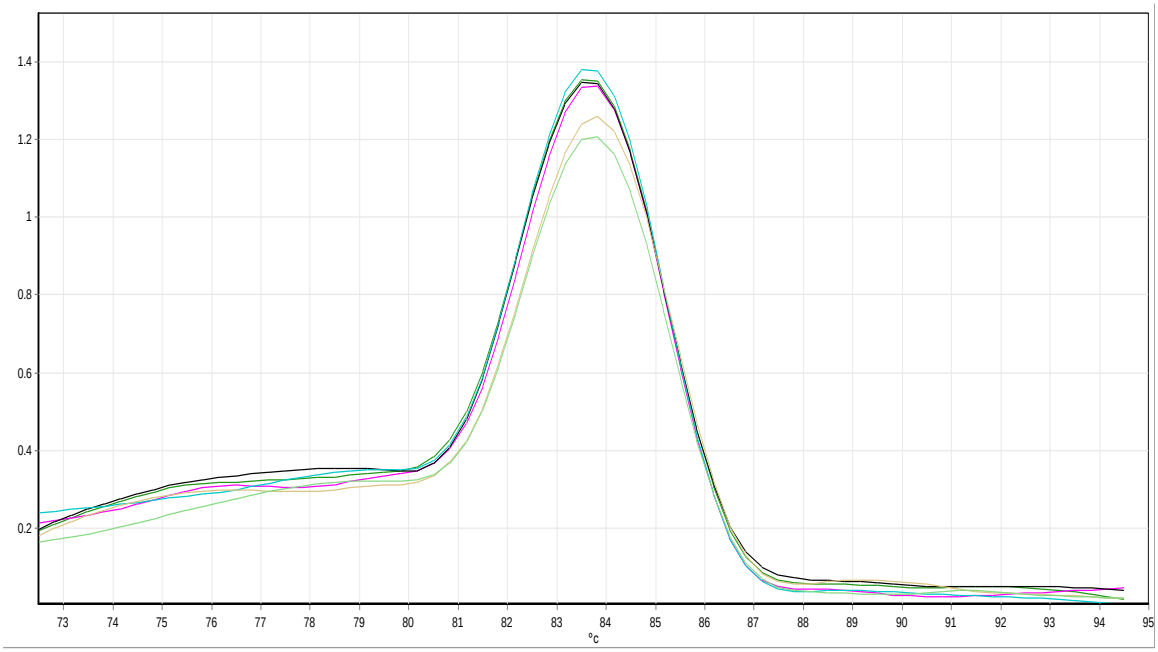


Figure 5.3: (**A**) GPR43 expression in HT-29 cell line, (**B**) Melt curve of the corresponding samples

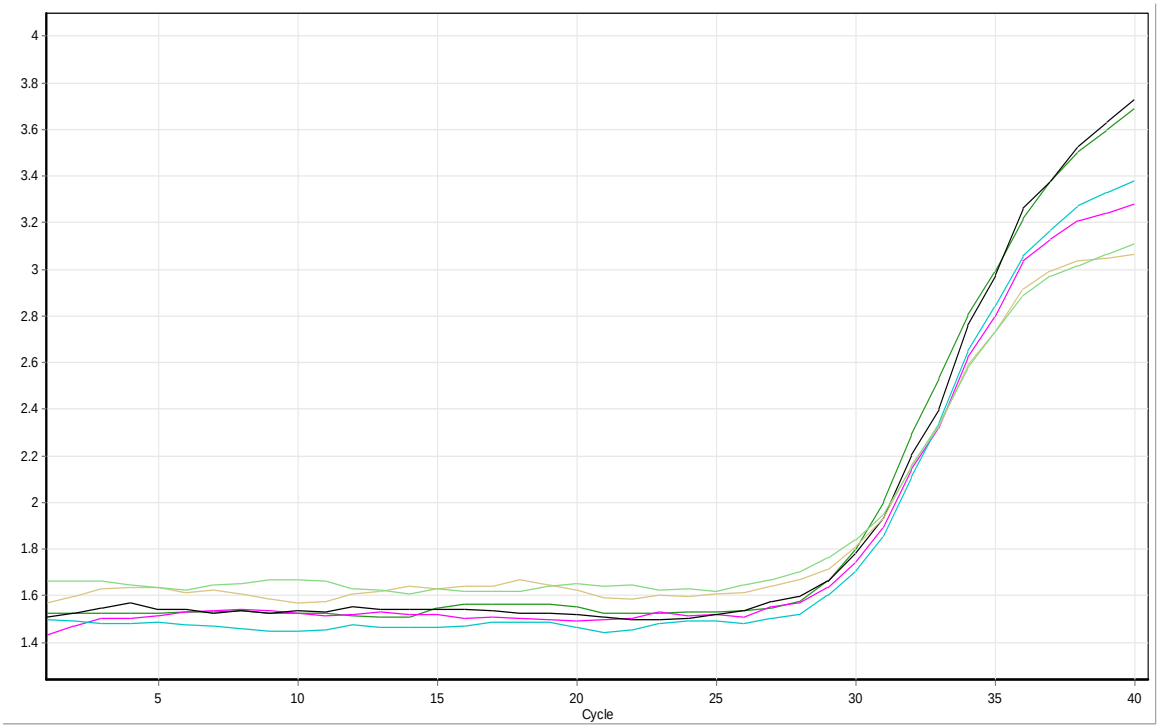


Figure 5.4: GPR41 expression in corresponding samples from HT-29 cell line

GPR43 expression was 14 fold more than GPR41 in HT-29 cell line (Ct value 28 vs. 35 respectively, where the threshold was set at 2.8 for comparative purposes) (Figures 5.3 & 5.4).

SW620

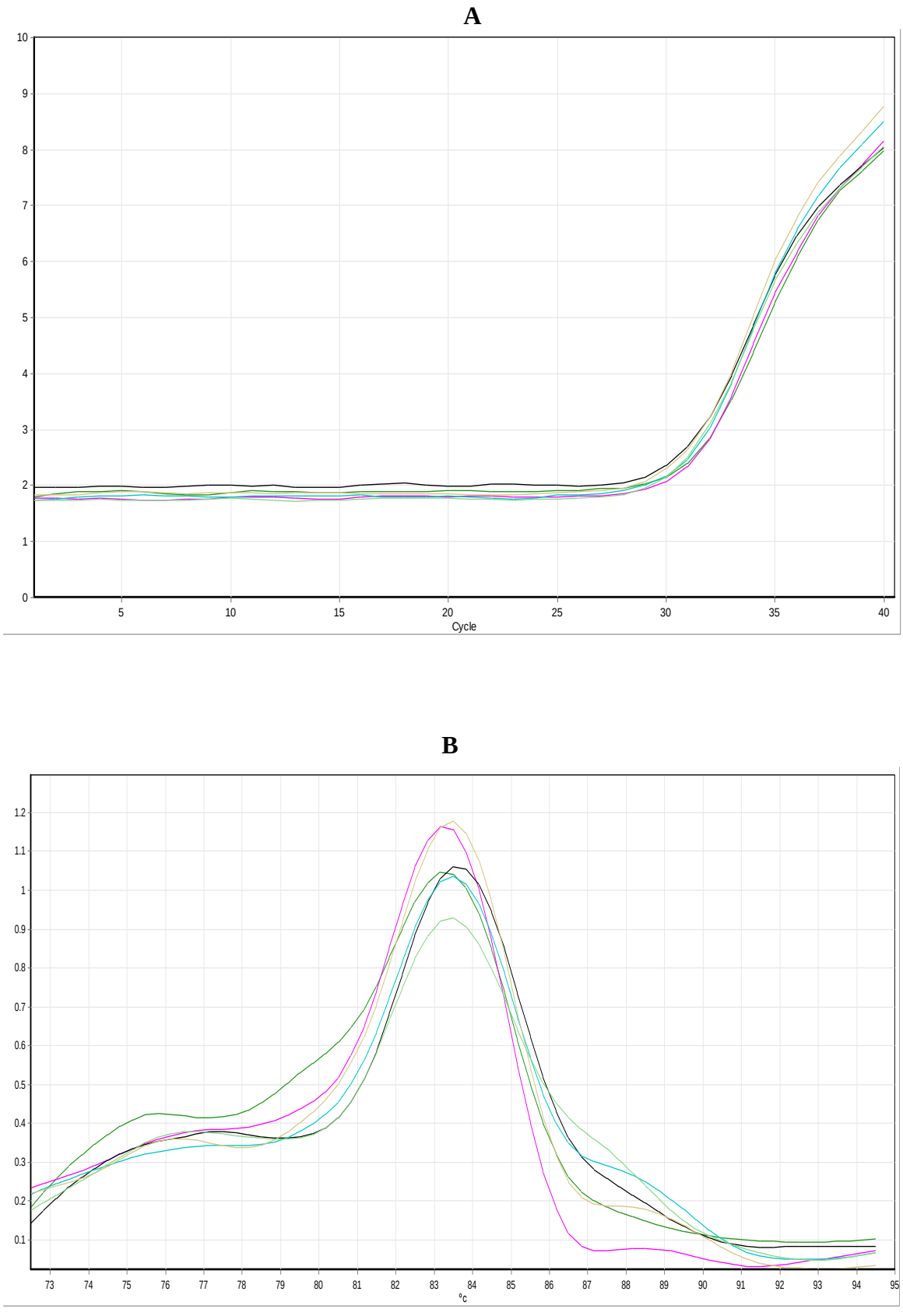


Figure 5.5: (**A**) GPR43 expression in SW620 cell line, (**B**) Melt curve of the corresponding samples

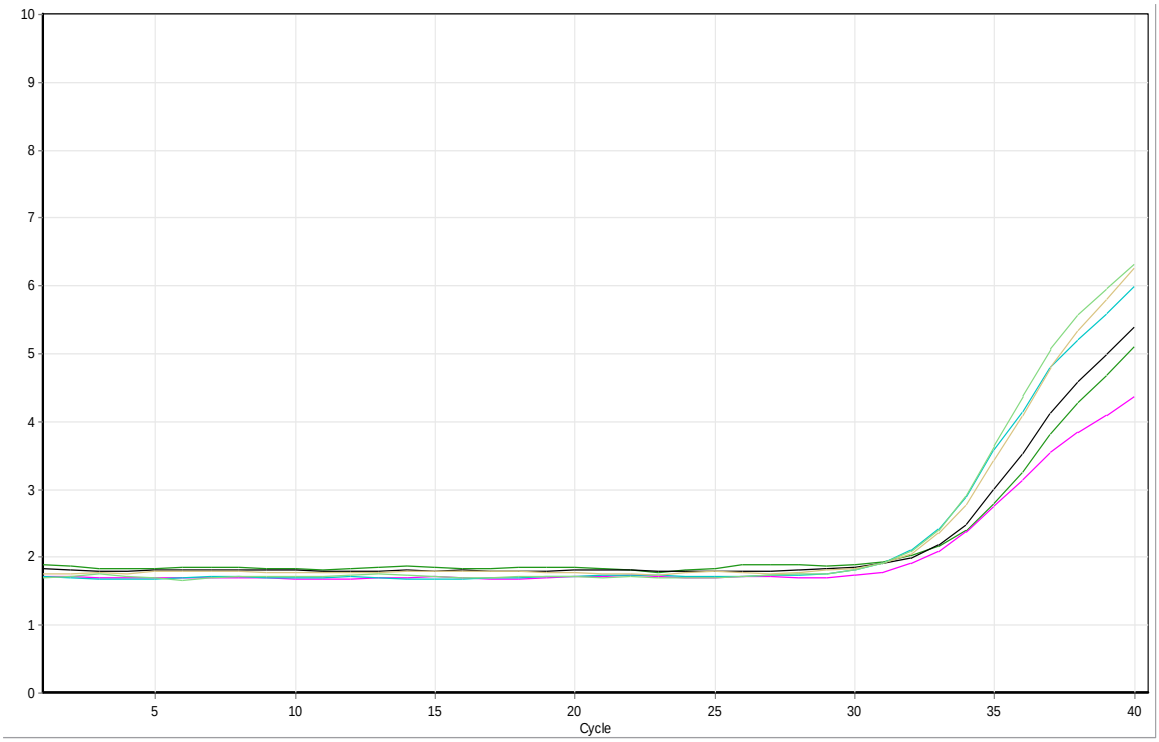


Figure 5.6: GPR41 expression in corresponding samples from SW620 cell line

GPR43 expression was 5 fold higher than GPR41 in SW620 (Ct value of 32 vs. 34.5 respectively, where the threshold was set at 3 for comparative purposes), see figures 5.5 & 5.6. GPR43 expression was 8 fold higher in HT-29 than in SW620 (Ct value of 28 vs. 32 respectively, where threshold set at 2.8), see figures 5.3A & 5.5A. HT-29 and SW620 had similar level of GPR41 expression (Ct value of 34 vs. 34.5 respectively), see figures 5.4 & 5.6.

5.3.3 Effects of butyrate on HT-29

GPR43

Butyrate exerted different effects in different cell lines. The results of two cell lines will be presented separately. At the concentration of 5 mM, GPR43 expression was significantly reduced by 9 fold, 14 fold, 29 fold, 35 fold at 2 hours, 4 hours, 6 hours and 12 hours respectively. Its negative effect was highest at 12 hours. There was a linear inverse relationship between the duration of butyrate incubation and the level of GPR43 expression (Table 5.1 & figure 5.7).

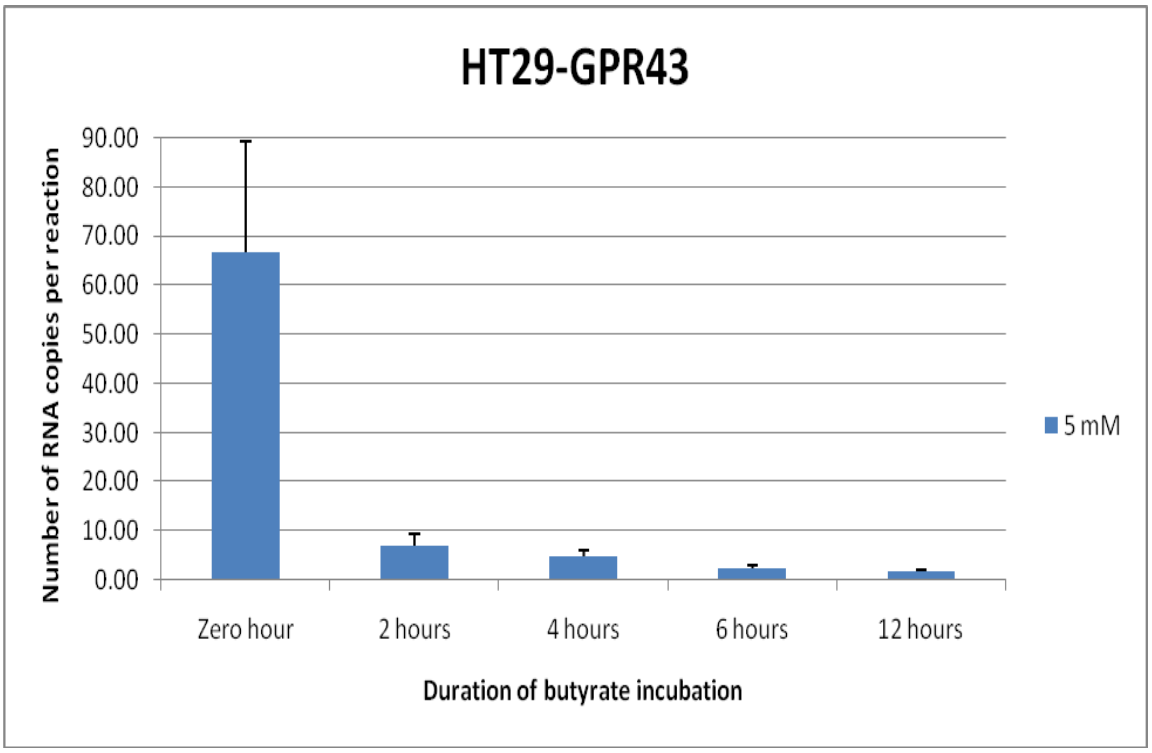


Figure 5.7: The effect of 5 mM of butyrate on GPR43 in HT-29 cell line, error bar indicates standard deviation

At the concentration of 10 mM, GPR43 expression was significantly reduced by 5 fold, 5 fold, 17 fold, 22 fold at 2 hours, 4 hours, 6 hours and12 hours respectively. Its negative effect was highest at 12 hours of incubation. There was a linear inverse relationship between the duration of butyrate incubation and the level of GPR43 expression demonstrated at 10 mM concentration (Table 5.1 & figure 5.8).

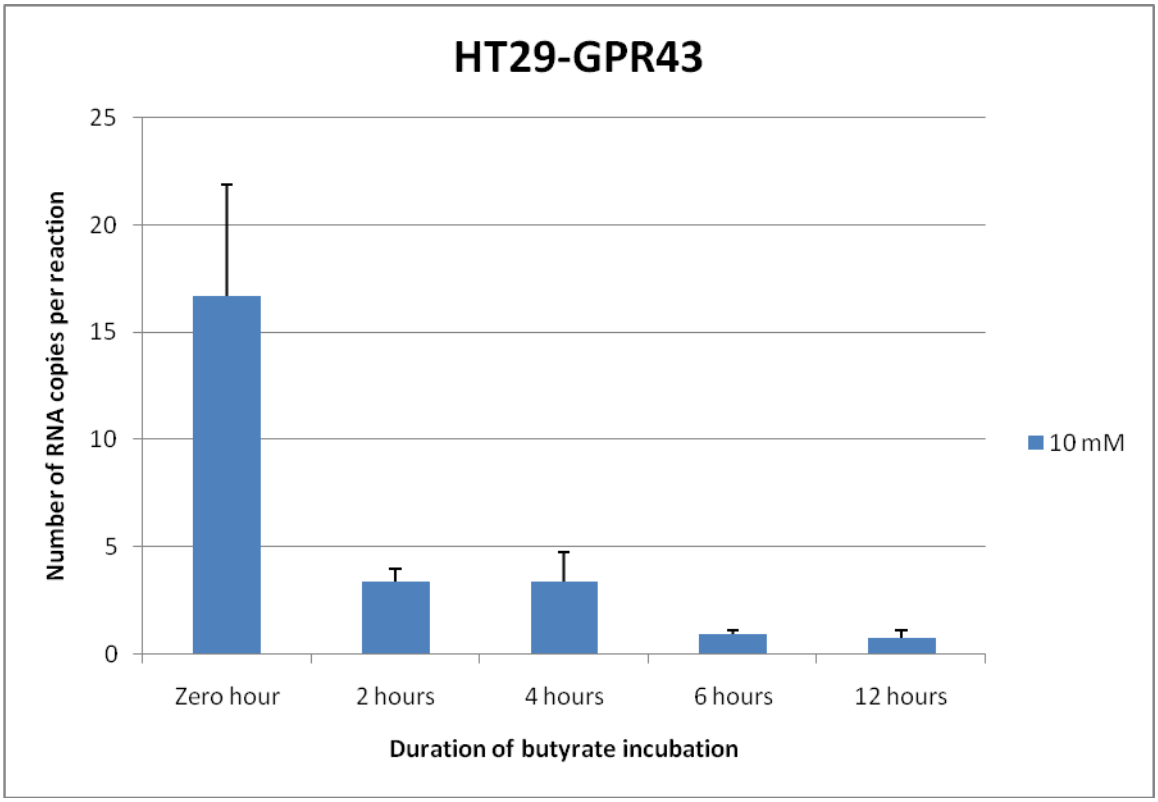


Figure 5.8: The effect of 10 mM of butyrate on GPR43 in HT-29 cell line, error bar indicates standard deviation

At 15 mM concentration, GPR43 expression was significantly decreased by 3 fold, 19 fold, 41 fold, 33 fold at 2, 4 , 6 and 12 hours respectively. The maximal negative effect was demonstrated at 6 hours of incubation with butyrate (Table 5.1 & figure 5.9).

	5 mM			**10 mM**			**15 mM**		
	Mean	**SD**	**Reduced in fold**	**Mean**	**SD**	**Reduced in fold**	**Mean**	**SD**	**Reduced in fold**
Zero hour	66.72	22.58		16.69	5.19		46.87	13.14	
2 hours	7.07	2.26	9*	3.40	0.60	5*	13.98	7.21	3*
4 hours	4.92	1.14	14*	3.41	1.33	5*	2.53	1.09	19*
6 hours	2.28	0.54	29*	0.96	0.20	17*	1.14	0.25	41*
12 hours	1.89	0.25	35*	0.77	0.32	22*	1.42	0.56	33*

Table 5.1: HT-29 GPR43, relative number of RNA copies followed incubation with butyrate, p value based on paired-samples t-test, * indicates statistical significant results, $p < 0.05$

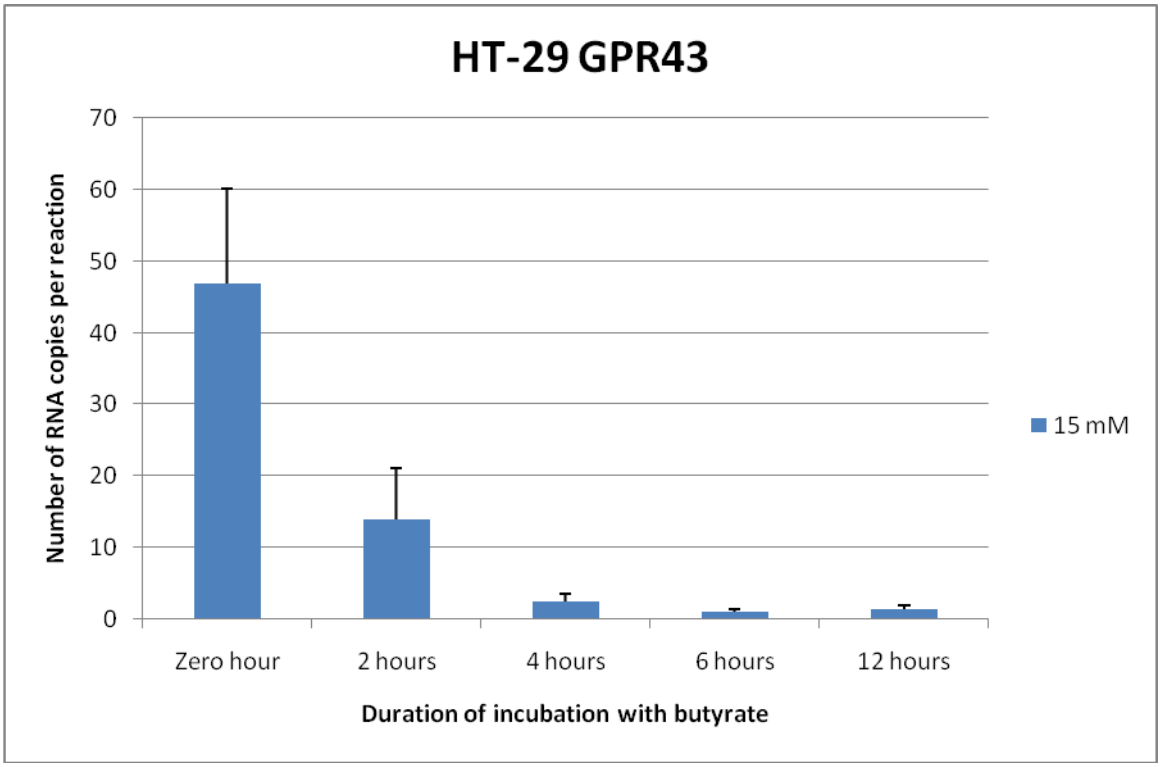


Figure 5.9: The effect of 15 mM of butyrate on GPR43 in HT-29 cell line, error bar indicates standard deviation

GPR41

At 5 mM concentration, GPR41 expression was increased at 2 hours by 1.4 fold. No changes were observed at 4 hours and there was a trend in reduction of expression demonstrated from 6 hours to 12 hours. The GPR41 expression was reduced by 2.7 fold, 5.3 fold at 6 and 12 hours respectively (Table 5.2 & figure 5.10).

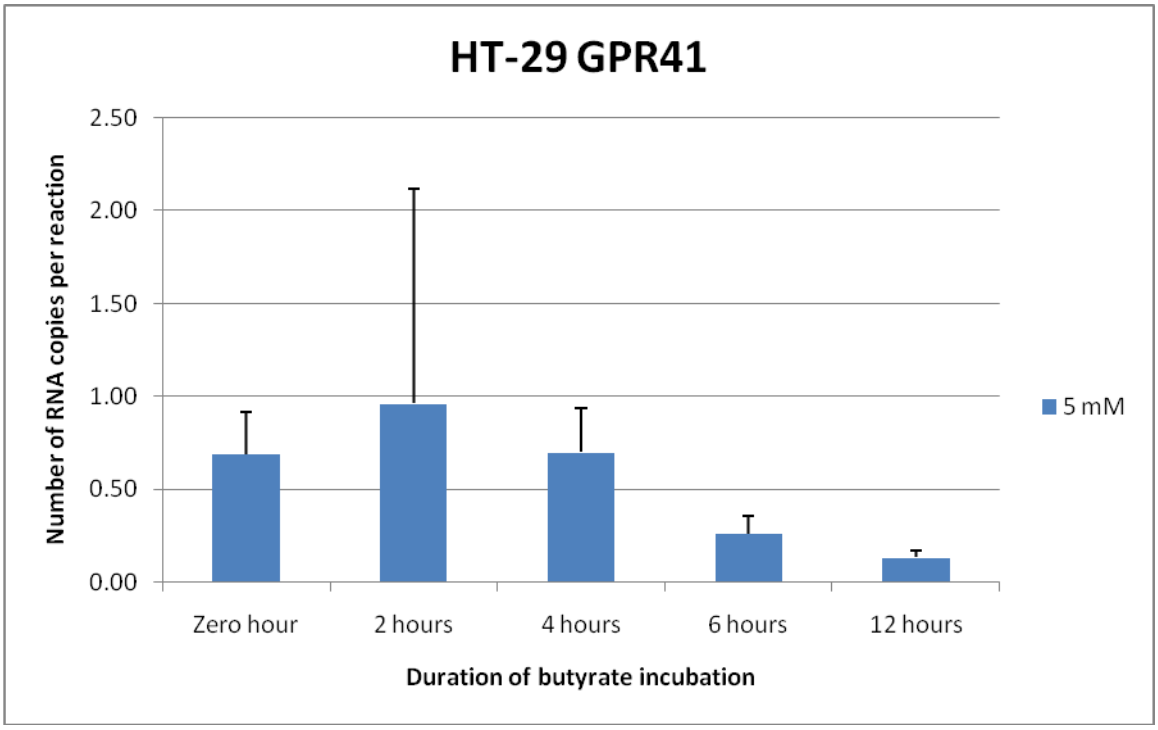


Figure 5.10: The effect of 5 mM of butyrate on GPR41 in HT-29 cell line, error bar indicates standard deviation

	5 mM			10 mM			15 mM		
	Mean	**SD**	**Changes in fold**	**Mean**	**SD**	**Changes in fold**	**Mean**	**SD**	**Changes in fold**
Zero hour	0.69	0.22		0.42	0.15		5.50	1.78	
2 hours	0.96	1.16	+1.4	0.28	0.19	-1.5	6.31	2.18	+1.1
4 hours	0.70	0.24	1	0.23	0.10	-1.8*	0.30	0.16	-18.3*
6 hours	0.26	0.10	-2.7*	0.10	0.06	-4.2*	0.22	0.15	-25*
12 hours	0.13	0.04	-5.3*	0.17	0.04	-2.5*	0.38	0.08	-14.5*

Table 5.2: HT-29 GPR41, relative number of RNA copies followed incubation with butyrate, p value based on paired-samples t-test, * indicates statistical significant results, $p < 0.05$

At 10 mM concentration, GPR41 expression was depressed by 1.5 fold, 1.8 fold, 4.2 fold and 2.5 fold at 2, 4, 6 and 12 hours of incubation. At 15 mM concentration, significant reduction in GPR41 expression was also observed from 4 hours and thereafter, the least expression was observed at 6 hours of incubation (Table 5.2, figures 5.11 & 5.12). The effect of butyrate of GPR41 in HT-29 cell line was not as marked in comparison to GPR43. The level of down regulation was not as significant and occurred randomly, there was no general trend of reduction in expression. Reduction in expression of GPR41 was observed at all the tested time frame of incubation at 10 mM concentration. The reduction was more marked at 6 hours of incubation at 15 mM concentration, similar finding for GPR43 expression (Figure 5.12)

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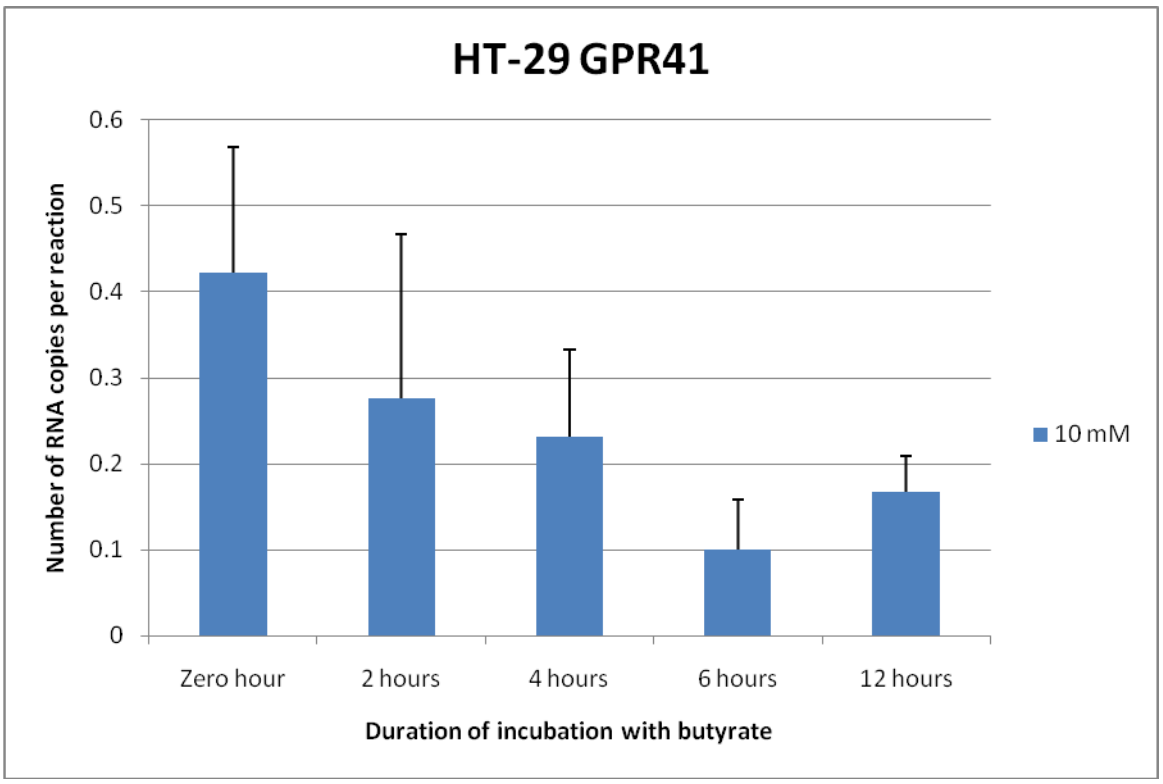

Figure 5.11: The effect of 10 mM of butyrate on GPR41 in HT-29 cell line, error bar indicates standard deviation

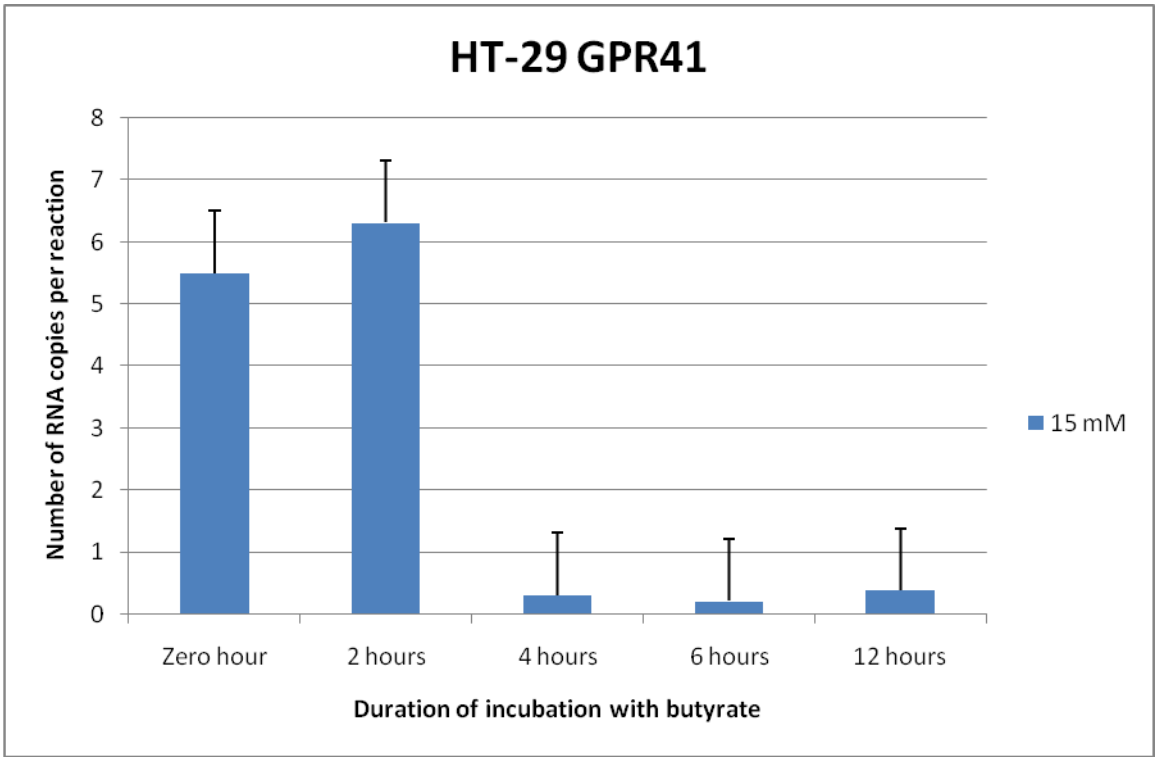

Figure 5.12: The effect of 15 mM of butyrate on GPR41 in HT-29 cell line, error bar indicates standard deviation

5.3.4 Effects of butyrate on SW620

GPR43

There was up-regulation of GPR43 expression in SW620 cell line at concentration of 5 mM of butyrate. GPR43 expressions were increased by 2 fold, 2 fold, 2 fold, 3 fold at 2, 4, 6 and 12 hours of incubation respectively. The highest level of expression was seen at 12 hours after incubated with butyrate (Table 5.3 & figure 5.13).

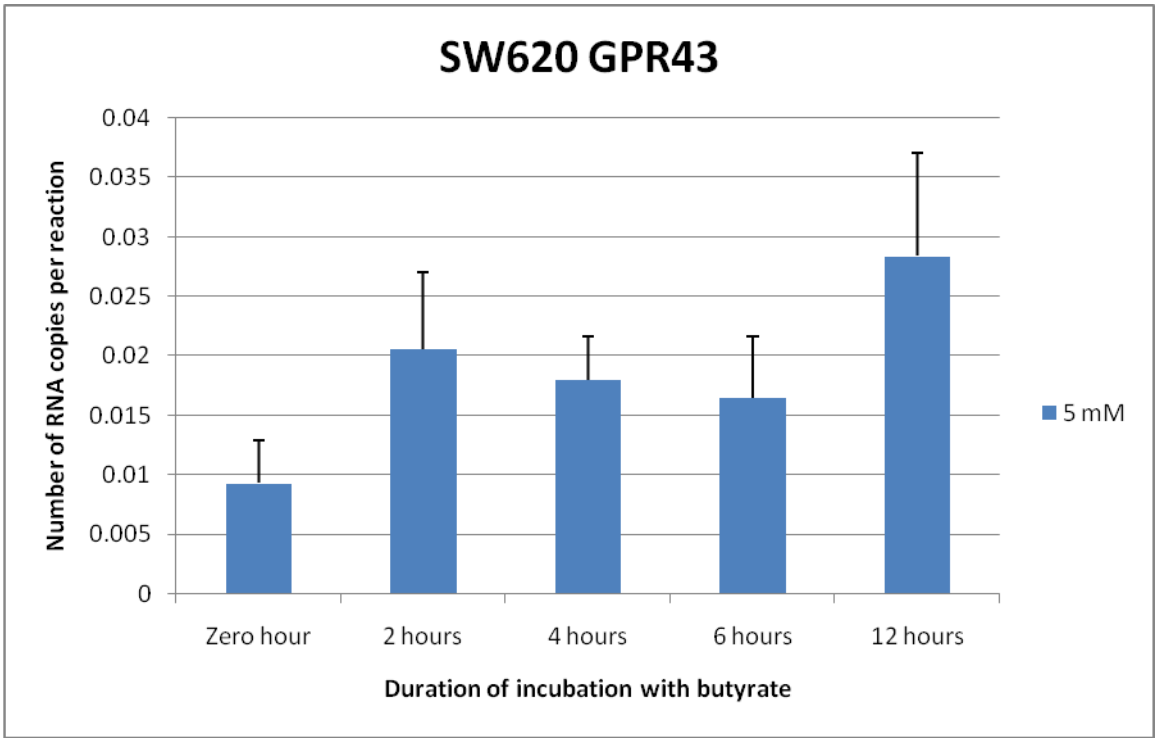

Figure 5.13: The effect of 5 mM of butyrate on GPR43 in SW620 cell line, error bar indicates standard deviation

At 10 mM concentration of butyrate, a general trend of up-regulation of GPR43 expression was observed at 4 hours and thereafter. The level of expression was slightly decreased at 2 hours of incubation. GPR43 expression was increased by 1.2 fold, 1.2 fold, 2.6 fold at 4, 6 and 12 hours respectively. The highest level of expression was found at 12 hours of incubation with butyrate (Table 5.3 & figure 5.14).

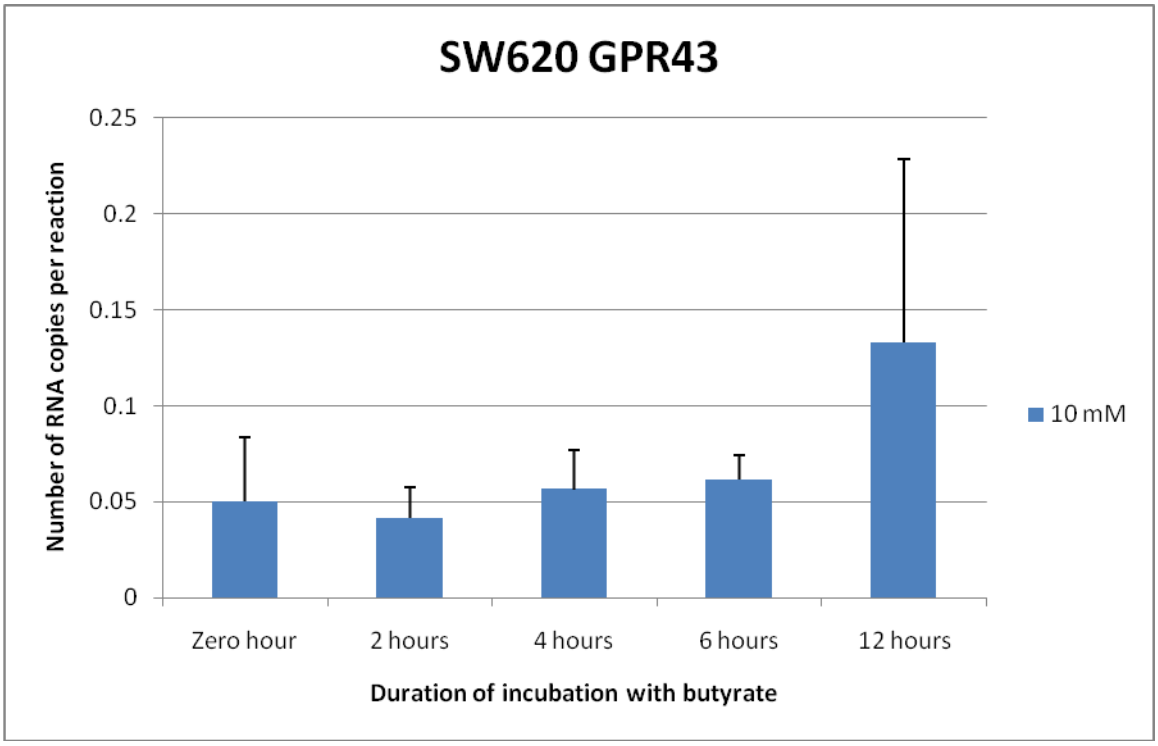

Figure 5.14: The effect of 10 mM of butyrate on GPR43 in SW620 cell line, error bar indicates standard deviation

	5 mM			10 mM			15 mM		
	Mean	**SD**	**Changes in fold**	**Mean**	**SD**	**Changes in fold**	**Mean**	**SD**	**Changes in fold**
Zero hour	0.01	0.004		0.05	0.03		0.17	0.05	
2 hours	0.02	0.01	+2*	0.04	0.02	-1.25	0.12	0.05	-1.4
4 hours	0.02	0.004	+2*	0.06	0.02	+1.2	0.14	0.05	-1.2
6 hours	0.02	0.01	+2	0.06	0.01	+1.2	0.19	0.10	+1.1
12 hours	0.03	0.01	+3*	0.13	0.10	+2.6*	0.14	0.04	-1.2

Table 5.3: SW620 GPR43, relative number of RNA copies followed incubation with butyrate, p value based on paired-samples t-test, * indicates statistical significant results, $p < 0.05$

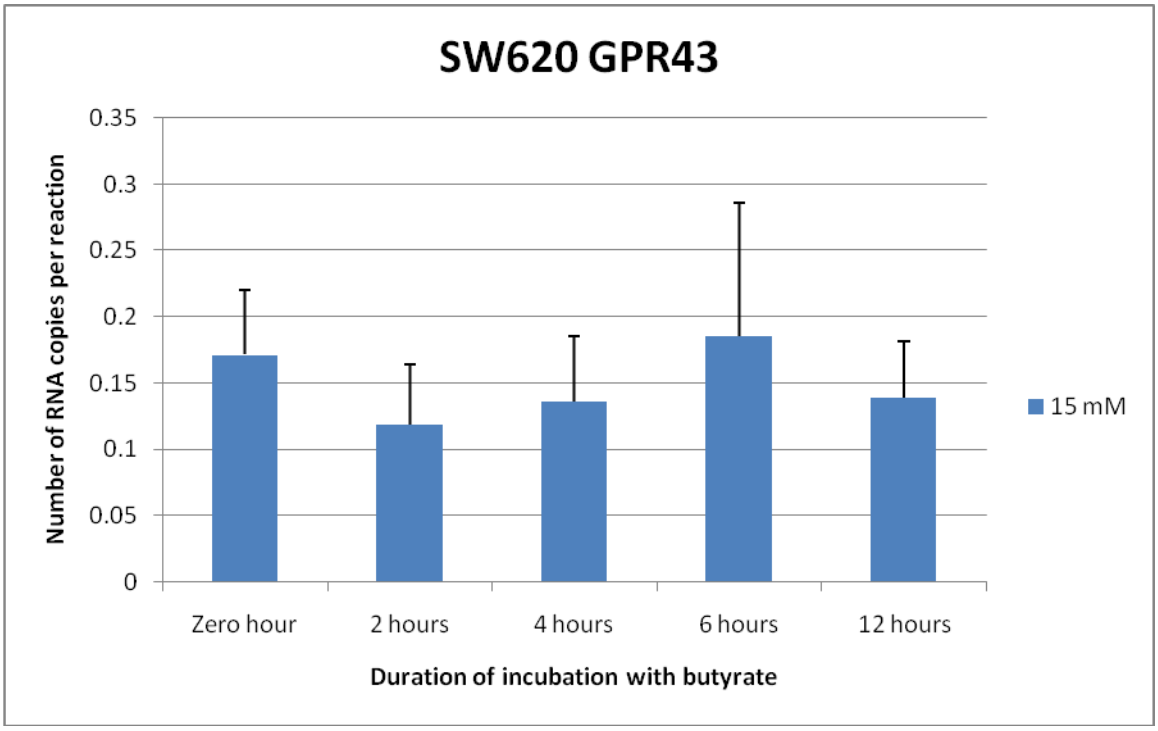

Figure 5.15: The effect of 15 mM of butyrate on GPR43 in SW620 cell line, error bar indicates standard deviation

At 15 mM of concentration, the responses were variable. GPR43 expression was reduced by 1.4 fold, 1.2 fold, 1.2 fold at 2, 4 and 12 hours respectively. However its expression was not significantly up-regulated at 6 hours by 1.1 fold. The highest level of GPR43 expression was observed at 6 hours of incubation (Table 5.3 & figure 5.15). Overall butyrate up-regulated GPR43 expression in SW620 at 5 and 10 mM concentration but the opposite effect was seen at 15 mM.

GPR41

GPR41 expression was generally increased at concentration of 5 mM of butyrate. GPR41 level were increased by 1.5 fold, 1.5 fold, 2 fold at 2, 4 and 12 respectively. There was only slight increase at 6 hours of incubation. The highest level of expression was found at 12 hours of incubation (Table 5.4 & figure 5.16).

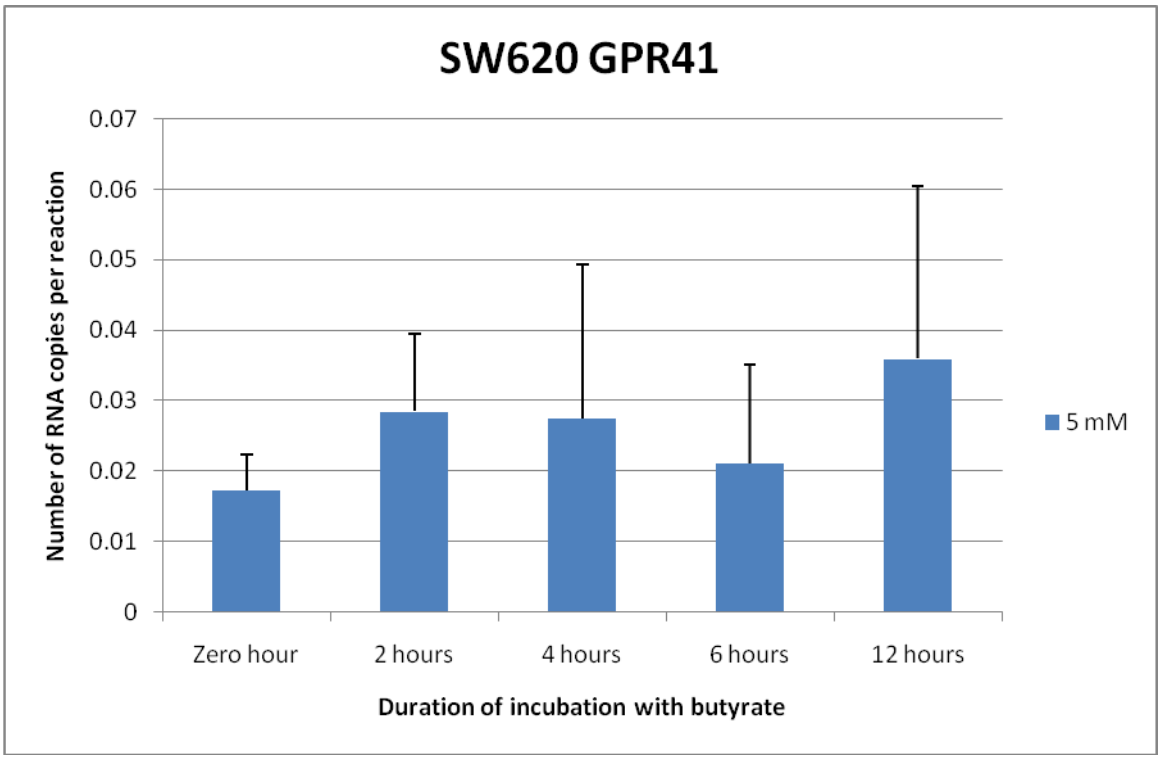

Figure 5.16: The effect of 5 mM of butyrate on GPR41 in SW620 cell line, error bar indicates standard deviation

	5 mM			10 mM			15 mM		
	Mean	**SD**	**Changes in fold**	**Mean**	**SD**	**Changes in fold**	**Mean**	**SD**	**Changes in fold**
Zero hour	0.02	0.01		0.05	0.02		0.02	0.01	
2 hours	0.03	0.01	+1.5*	0.03	0.02	-1.7	0.05	0.04	+2.5
4 hours	0.03	0.02	+1.5	0.03	0.01	-1.7*	0.02	0.01	1
6 hours	0.02	0.01	1	0.04	0.03	-1.25	0.01	0.01	-2
12 hours	0.04	0.02	+2	0.07	0.05	+1.4	0.03	0.02	+1.5

Table 5.4: SW620 GPR41, relative number of RNA copies followed incubation with butyrate, p value based on paired-samples t-test, * indicates statistical significant results, $p < 0.05$

GPR41 expression was decreased at 2 hours of incubation but the level of expression was gradually increased and peaked at 12 hours at 10 mM concentration. The level of GPR43 was decreased by 1.75 fold, 1.75 fold, 1.25 fold at 2, 4 and 6 hours respectively. In contrast, it was increased by 1.4 fold at 12 hours (Table 5.4 & figure 5.17).

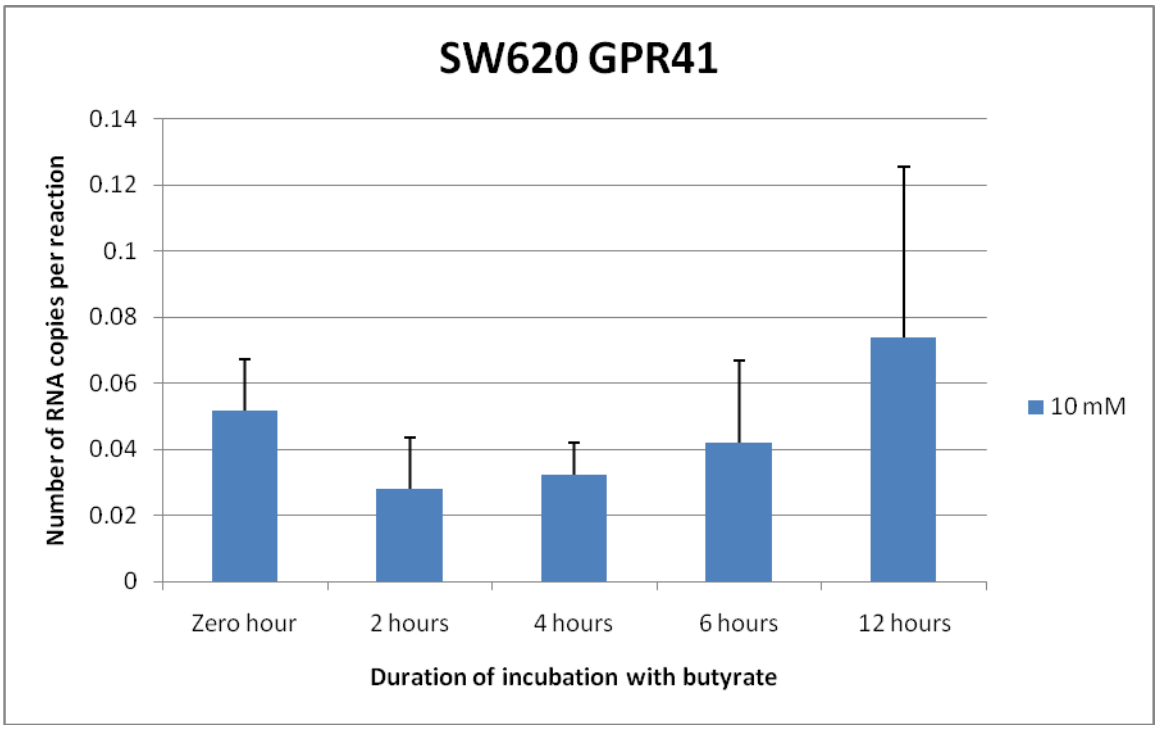

Figure 5.17: The effect of 10 mM of butyrate on GPR41 in SW620 cell line, error bar indicates standard deviation

GPR41 expression was erratic at 15 mM of butyrate. There was no general trend in either increasing or decreasing. The level was higher by 2.5 fold, 1.5 fold at 2 and 12 hours respectively. There was no significant difference at 4 hours and decreased by 2 fold at 6 hours (Table 5.4 & figure 5.18).

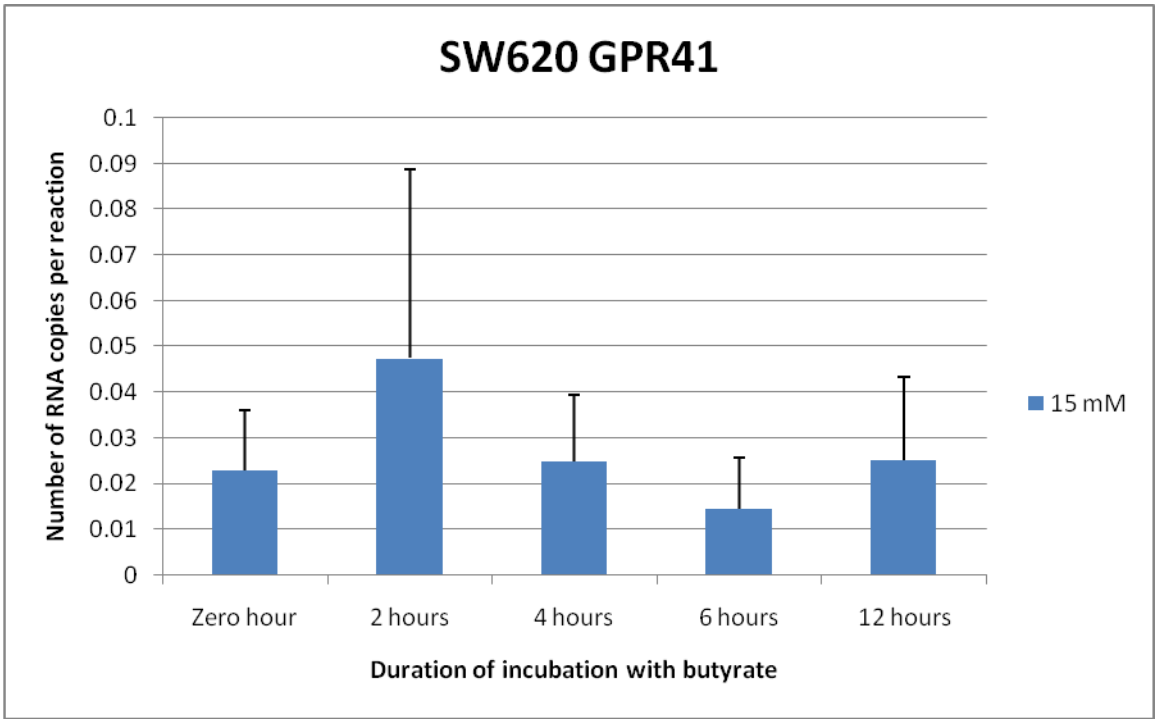

Figure 5.18: The effect of 15 mM of butyrate on GPR41 in SW620 cell line, error bar indicates standard deviation

Butyrate overall had an up-regulation effect on GPR41 in SW620 cell line. The effect was marginal with 2 to 2.5 fold increase, the highest level of expression was observed at 12 hours of incubation at 5 and 10 mM concentrations. At 15 mM concentration the highest level of expression was observed at 2 hours of incubation.

5.3.5 Comparison between concentrations vs. duration of incubation

HT-29

GPR43 was statistical significantly down regulated in HT-29 cell line as the result of incubation with butyrate. This effect was demonstrated at 5 mM, 10mM and 15 mM concentrations. There was an obvious linear trend of down regulation at 5 mM concentration of butyrate. GPR43 was expressed the least at 12 hours of incubation across three levels of concentrations. The level of suppression correlated to the concentration of butyrate, the greater the concentration the greater the level of down regulation. The longer the incubation time, the greater the suppression effect and peaked at 12 hours (Tables 5.1& 5.2 and figure 5.19).

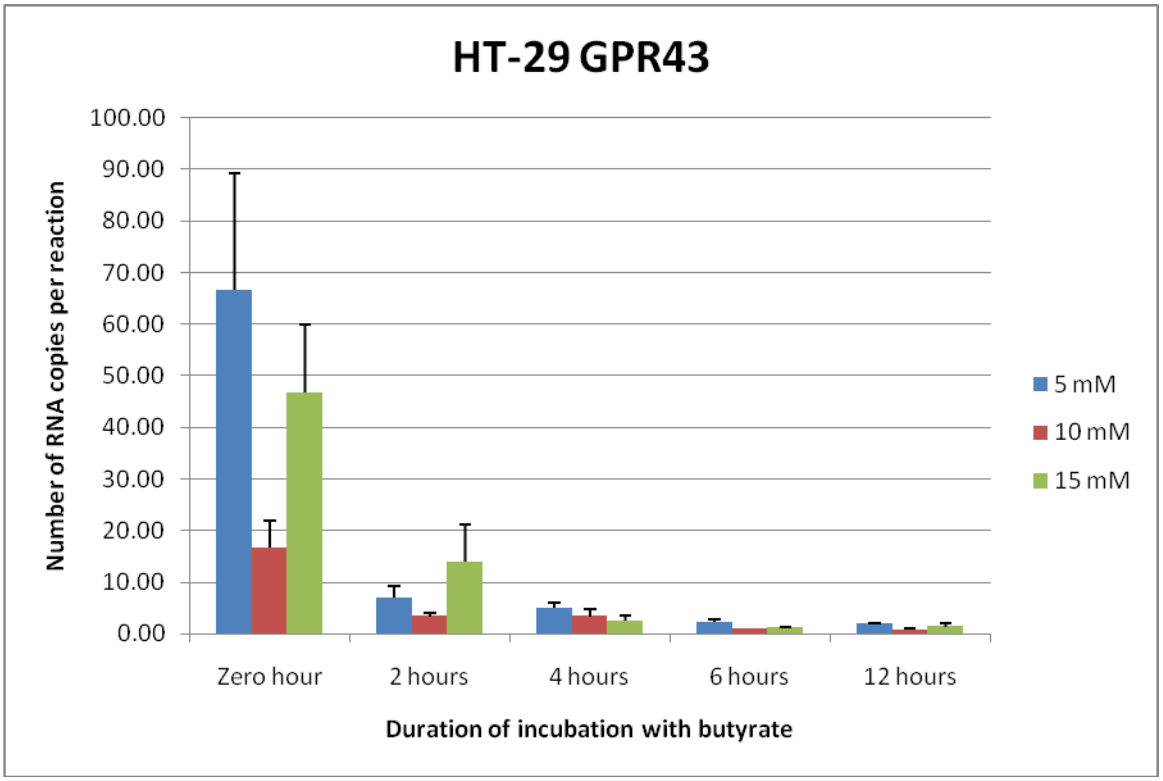

Figure 5.19: The effect on GPR43 in HT-29 cell line, comparison of 3 concentrations at different incubation time, error bar indicates standard deviation

In contrast to GPR43, the effect on GPR41 was not as marked, the down regulation effect was not seen until 4 hours for 5, 10 and 15 mM concentrations. The level of expression was higher at 2 hours. Overall there was a trend of down regulation of GPR41. The degree of down regulation was directly related to the concentration and duration of incubation, the higher the concentration the greater the effect of down regulation likewise for the duration of incubation (Table 5.2 & figure 5.20).

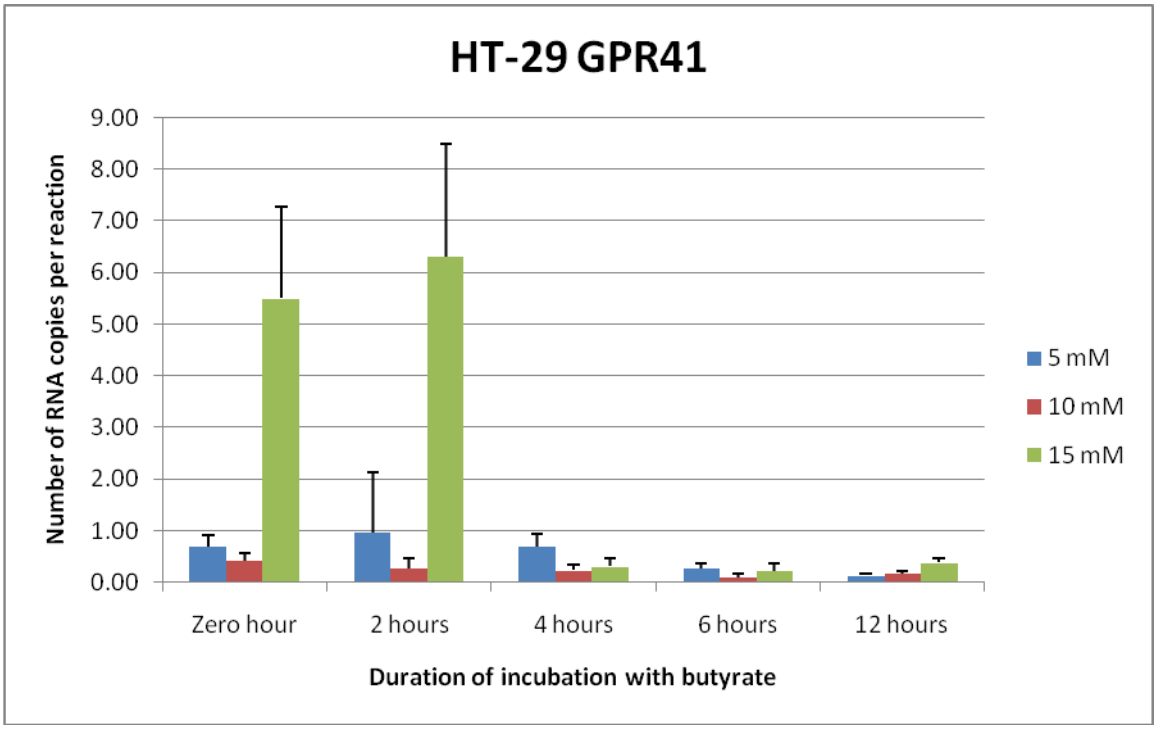

Figure 5.20: The effect on GPR41 in HT-29 cell line, comparison of 3 concentrations at different incubation time, error bar indicates standard deviation

SW620

The opposite effect was observed in SW620 cell line, butyrate up regulated the expression of GPR43. The up regulation effect occurred in all three tested concentrations however there was no increasing trend with increasing concentration of butyrate. The highest level of expression was observed at 12 hours of incubation across 3 concentrations. The number of RNA copies was significant less than those in HT-29 cell line under the same condition and concentration (Table 5.3 & figures 5.19, 5.21).

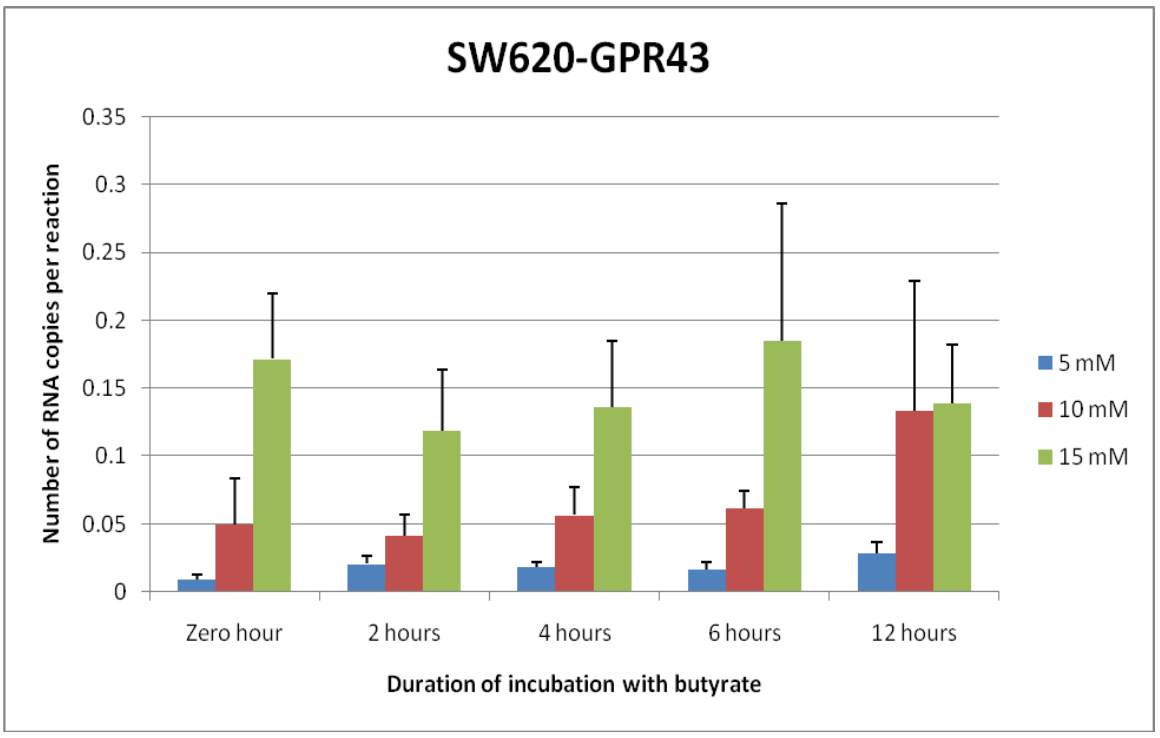

Figure 5.21: The effect on GPR43 in SW620 cell line, comparison of 3 concentrations at different incubation time, error bar indicates standard deviation

Butyrate had variable effect on GPR41 in SW620 cell line. There was a trend of up regulation of GPR41 expression but it did not seen to correlate with increasing concentration nor increasing duration of incubation. The level of expression was lowest at 6 hours of incubation and highest at 12 hours (Table 5.4 & figure 5.22).

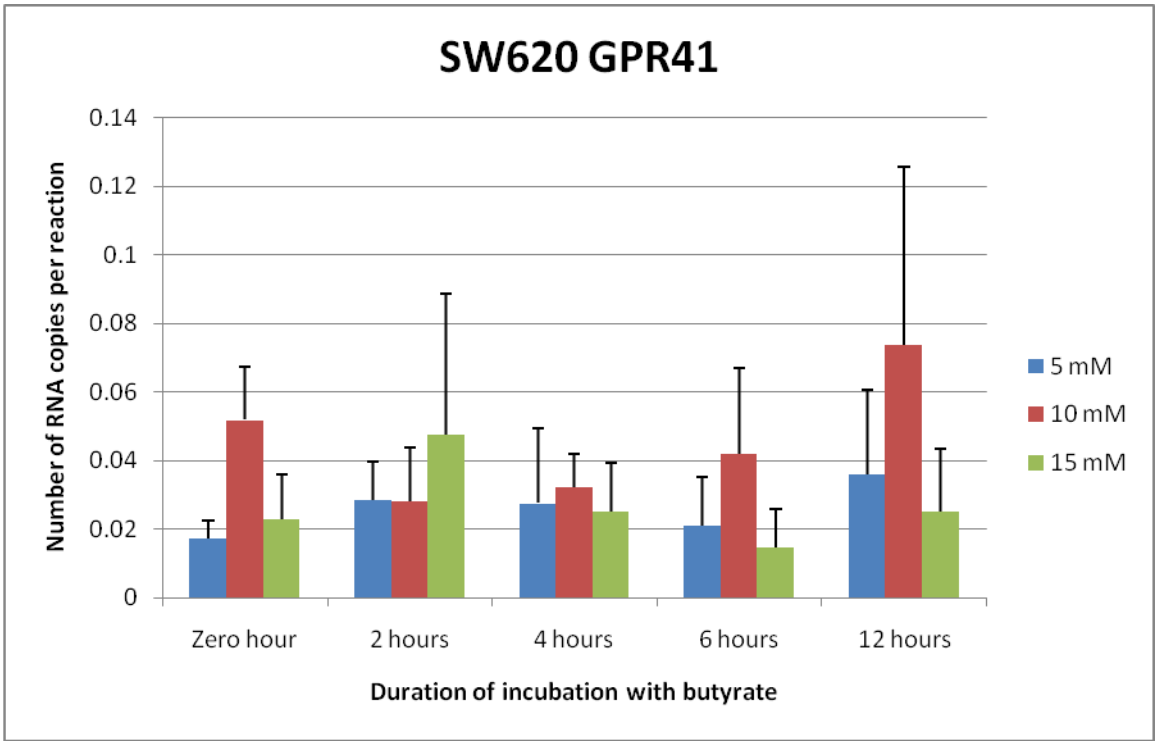

Figure 5.22: The effect on GPR41 in SW620 cell line, comparison of 3 concentrations at different incubation time, error bar indicates standard deviation

5.4 Discussion

Cancer cell lines are immortal. There are many commercially available cancer cell lines including colonic cancer cell lines. Unfortunately, there is no rectal cancer cell line available for purchase. Since the intraluminal concentration of butyrate is least in the rectum, rectal cancer cell lines would be very attractive to research. The advantage of commercially available colonic cell lines is that experiments can be repeated by different research groups to validate others' results.

HT-29 and SW620 colorectal cancer cell lines were selected primarily because they are available commercially and also because a great amount of work and research has been conducted on these cell lines. The findings of these studies provided the foundation for future research into GPR43 and GPR41, in the hope of finding a cure or prevention for human colorectal cancer.

There is no literature regarding GPR43 and GPR41 expression in colorectal cancer cell lines. The present study is among the early literature. Subsequent to demonstrating that colorectal cancer did express GPR43 and GPR41, expression of GPR43 and GPR41 in colorectal cancer cell lines HT-29 and SW620 was examined. Similar to normal and tumour colorectal epithelium, GPR43 expression was higher than GPR41. It was more significant in HT-29 than SW620, with 14 fold and 5 fold increases respectively, whereas the level of GPR41 expression was similar in both cell lines. It was possible to compare real-time RT-PCR directly because experiment conditions were the same for both cell lines. Interpersonal variation in gene expression is an obstacle in human tissue molecular biology experiments. The molecular basis for the difference is unknown. Because the present study is among the pioneers in assessing the effect of butyrate on colonic cancer cell lines, results could not be compared to those of other researchers. More research is needed, therefore, to validate these findings.

It is not possible to conduct in vivo colorectal cancer experiments with butyrate. Cancer develops over a long period of time and it is unethical to expose human subjects to carcinogens. In vitro cancer research is indirect, mainly via cancer cell lines prior to human clinical trials. An advantage is that such studies can be designed in such a way that the environment can be modified and maintained to test the hypotheses. In addition, many confounding factors such as luminal concentration of butyrate cannot be controlled with in vivo experiments. It is unethical to conduct similar studies to assess the effects of butyrate on live human subjects because not only would it require surgical resection of the bowel in otherwise healthy human subjects, but the anti-neoplastic effects of butyrate have not been consistently proven.

Immunohistochemistry staining of cell lines would provide more details about the nucleus receptors status of GPR43 and GPR41 but this was not technically possible. Attempts at fixation of cells pellets

with formalin failed. The cells pellets disintegrated upon placement in formalin solution. Attempts at mechanical removal of cells from culture flask resulted in cell death and distortion of cell architecture.

As demonstrated here, different cancer cell lines have different biological properties. HT-29 and SW620 are both colonic cancer cell lines but each expresses a different level of GPR43 and GPR41. In reality, multiple experiments with many different cancer cell lines are recommended. One can neither conclude that butyrate down regulates expression of GPR43 and GPR41 nor confirm the hypothesis because this down regulation effect was only observed in the HT-29 cell line. In fact the reverse was seen in the SW620 cell line.

Significant differences in response to the effects of butyrate in HT-29 and SW620 highlights the fact that signal transduction processes and cell metabolism may be different in transformed cells such as colorectal cancer cell lines, compared to normal cells. Suspected down regulation of GPR43 and GPR41 by butyrate is likely to be true because chapter 3 has shown that there was less positive nucleus staining in tumour cells than in normal cells.

Future direction for further research should include a larger number of subjects. The use of laser-capture microdissection device would enhance the targeted sampling of tumour tissue and hence the RNA. The effects of butyrate when GPR43 and GPR41 are blocked should be assessed in the HT-29 and SW620 cell lines. The effects of butyrate on $p21^{Waf1/Cip1}$ in either functioning or blocking GPR43 and GPR41 should be assessed.

5.5 Thesis Summary

Dietary fiber studies show contradictory results, with some promoting a protective role against colorectal cancer while others find no such protective effects. Among several possible reasons for negative studies are a too low quantity of dietary fiber and a too short duration of study to show any protective effects. In some subjects, particularly older subjects, carcinomatosis has already commenced and butyrate from dietary fiber cannot reverse the process.

The protective effects of butyrate in preventing colorectal carcinogenesis have not been proved in animal studies because surrogate markers of carcinoma were used such as aberrant crypt foci and the amount of faecal butyrate rather than the butyrate concentration. Whereas in cell line experiments, environment, pH level and concentration of butyrate are all well maintained and controlled. Intra-luminal pH in the colon varies depending on fermentation of dietary fiber. Concentration of butyrate also varies depending on location of colonocytes within the crypt lumen. The conditions of this study cannot be replicated in experiments on animal or human subjects.

Butyrate is known to competitively inhibit histone deactylase (Riggs et al. 1977). Not until two decades later was butyrate found to induce apoptosis (Boisteau et al. 1996; Conway et al. 1995). Butyrate inhibits cell proliferation by stopping cell division at G_0/G_1 phase of cell division in HT-29 cell lines (Coradini et al. 2000). The inhibition effect is observed at as low concentration as 1mM. A solid body of evidence in the literature suggests that butyrate's proliferative inhibitory property is related to up regulation and down regulation of genes and oncogenes involved in cell proliferation (Krupitza et al. 1996). Doglioni et al. (1996) found that $p21^{Waf1/Cip1}$ expression is inversely related to proliferation and associated with terminal differentiation. This up regulation of $p21^{Waf1/Cip1}$ expression could be regulated either by p53 dependent and p53 independent pathways. There exists a substantial body of evidence in relation to $p21^{Waf1/Cip1}$ up regulation by butyrate. No attempt was made to assess the effects of butyrate on $p21^{Waf1/Cip1}$ in this cell line study, as it has been consistently shown in many other studies (Archer et al. 2005; Chen et al. 2004; Huang et al. 2005; Rocchi et al. 2005; Siavoshian et al. 2000; Wilson et al. 2006).

Normal cell cycle division involves a transient increased in $p21^{Waf1/Cip1}$ expression in the early G_1 phase as the result of transient activation of extracellular signal-related kinase and Ras mitogen-activated protein kinase pathways from the mitogenic signals (Bottazzi et al. 1999). The mitogenic signals and transient increase in $p21^{Waf1/Cip1}$ gene expression promote assembly of active cyclin D-dependent (D1-Cdk4/6) kinases and inhibit activity of cyclin E-dependent (E-Cdk2) kinase complex. An unknown mechanism leads to reduction of $p21^{Waf1/Cip1}$. Sequestration of $p21^{Waf1/Cip1}$ reduces the inhibitory effect

and allows activation of cyclin E-Cdk2 complex. The activated cyclin D- and E-dependent kinases are involved in phosphorylating Rb (Retinoblastoma protein). Rb hyper-phosphorylation results in disassociation of the Rb-E2F complex, allowing transcription factor E2F to stage the coordination of transcription of genes necessary for DNA synthesis in the S phase (Davie 2003; Sherr & Roberts 1999). Butyrate induces expression of $p21^{Waf1/Cip1}$, maintaining the inhibitory effect on the cyclin E-dependent (E-Cdk2) kinase complex which subsequently arrests the cell division at the G_1 phase. (See chapter 1.4 and figure 1.5). This model lacks the mechanism explaining/describing how butyrate induced $p21^{Waf1/Cip1}$ expression is regulated.

Richon et al. (2000) proposed that butyrate induces cell apoptosis by inducing $p21^{Waf1/Cip1}$ gene expression from inhibiting HDAC. This model is incomplete in failing to account for the presence of high quantities of butyrate in human colorectal lumen. It also fails to explain how butyrate is delivered to the nucleus, how butyrate is regulated or the mechanism by which the apoptotic process can be modulated in normal colonocytes function and proliferation, and expression of butyrate receptors (GPR43 and GPR41). The existence of butyrate receptors across many different types of human tissue, in particular human colorectal epithelium, raises many questions as to how butyrate induces apoptosis and its role in colorectal carcinogenesis.

The most recent work on short chain fatty acids and restored GPR43 expression in HCT8 colonic cancer cell by Tang et al. (2011) found that propionate can also induce apoptotic cell death by increased activation of initiator caspases 6,7 and 8 and common executioner caspase 3, and decreased expression of anti-apoptotic Bcl-2 and Survivin proteins. It is possible that butyrate can mediate via GPR43 and similar effects are expected. Earlier studies also suggest butyrate initiates apoptosis via mitochondrial pathway (Heerdt, Houston & Augenlicht 1997; Wang, Luo & Xia 2009). An alternative model as to how butyrate induces apoptotic effects is proposed. The binding of butyrate to the nucleus membrane or intra-nucleus GPR43 and GPR41 induces expression of $p21^{Waf1/Cip1}$. The increased $p21^{Waf1/Cip1}$ level results in cell arrest. Activation of cell membrane GPR43 and GPR41 results in initiation of the intrinsic apoptotic pathway, leading to irreversible apoptosis (Figure 5.23).

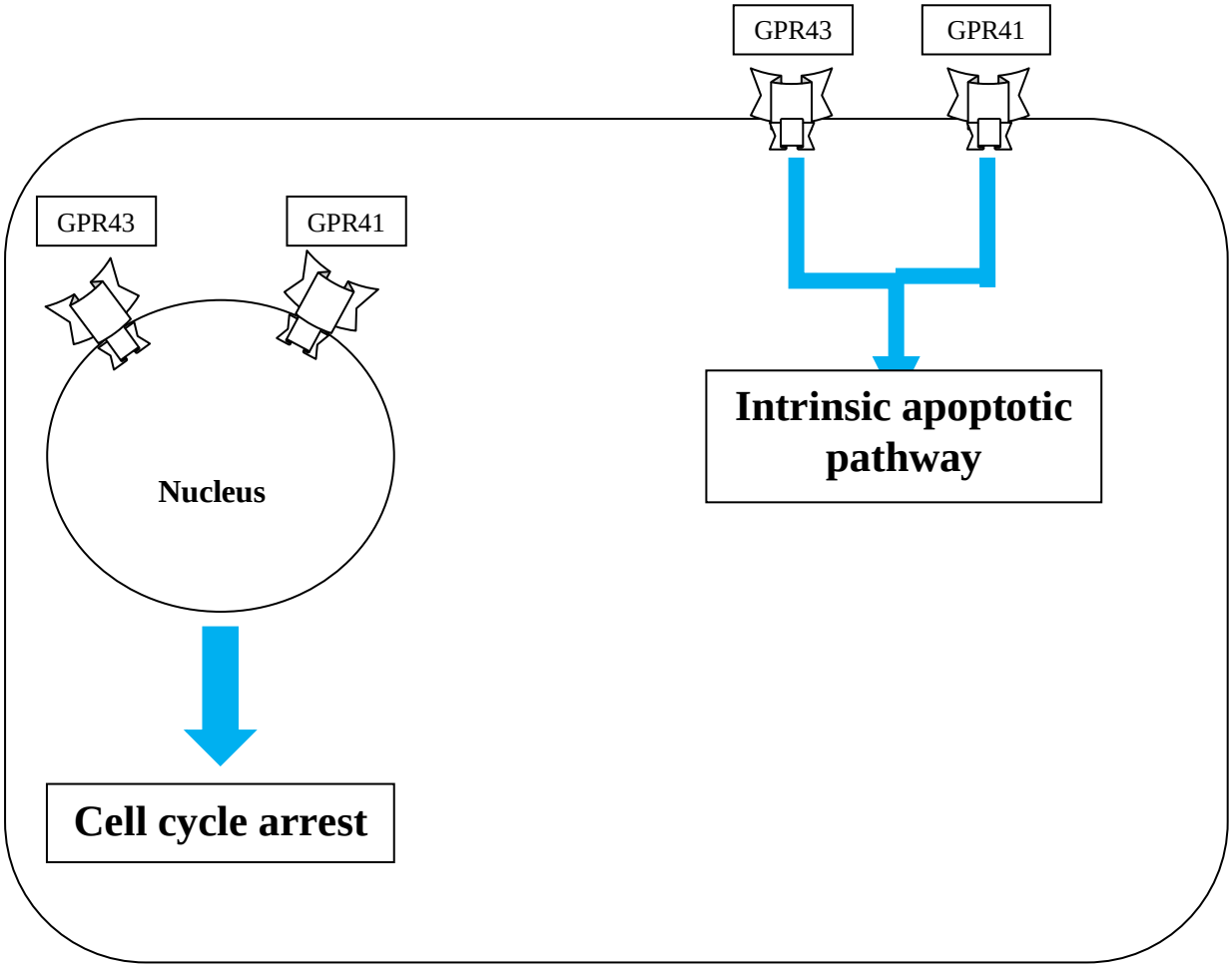

Figure 5.23: The proposed alternative model of how butyrate exerts its apoptotic effects via GPR43 and GPR41

5.6 Conclusion

The findings of this paper showed that GPR43 and GPR41 were nucleus membrane receptors which were expressed more in normal colonocytes than tumour by immunohistochemistry and that butyrate significantly down regulated expression of GPR43 and GPR41 in HT-29 colonic cancer cell line. This down regulation effect was dependent on the duration of incubation and the concentration of butyrate. GPR43 and GPR41 can have a role in cell apoptosis but more research is needed to further evaluate these roles.

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