CHAPTER TWO: METHODS AND MATERIALS

2.1 Materials

Cancerous and normal colon tissue sections embedded in paraffin wax were provided by Dr Stewart Goetsch (Wits Medical School Dept of Anatomical Pathology). Informed consent of the patient was obtained prior to biopsies being obtained. 4 control subjects (patients without colon cancer) and 30 patient cases were used.

The colon cancer cell line HT29 was kindly donated by Dr Clem Penny from Wits Medical School. The normal kidney cell line Graham 293 was obtained from Highveld Biological.

Ethics clearance and higher degree approval were obtained from the University of the Witwatersrand.

2.2 Methods: RNA extraction

The Roche High Pure RNA Isolation Kit was used in conjunction with cultured cell lines:

1. The cells were resuspended in 200µl PBS.
2. 400µl of lysis/-binding buffer was added and the contents mixed well.
3. A High Pure filter tube and collection tube was set up and the sample pipetted into the filter tube.
4. This was centrifuged for 15 seconds at 10 000 rpm and the flowthrough discarded.

5. 90µl of DNase incubation buffer and 10µl of DNase I were mixed in a sterile tube and added to the solution in the filter tube. This was left to incubate for 15 minutes at 15-25°C.
6. 500µl of wash buffer I was added to the filter tube and centrifuged at 10 000 rpm for 15 seconds. The flowthrough was discarded.
7. 500µl of wash buffer II was added to the filter tube, centrifuged at 10 000 rpm for 15 seconds and the flowthrough discarded.
8. 200µl of wash buffer II was added to the filter tube, centrifuged at maximum speed for two minutes and the flowthrough discarded.
9. The filter tube was inserted into a sterile 1.5ml reaction tube. To this, 50-100µl of elution buffer was added and centrifuged at 10 000 rpm for one minute.

2.3 Quantitative PCR

cDNA was synthesised from the RNA extracted in 2.2 above. Real-time PCR was then performed using the Roche LightCycler 2.0 System (Roche Diagnostics, Mannheim, Germany) to observe DNA amplification. This was done to quantify the degree to each DWNN transcript was expressed in a cancerous colon cell line (HT-29) compared to a normal cell line (Graham 293). This was accomplished by using primers designed to synthesize the DNA of each transcript.

Table 2.1 cDNA synthesis (RT)

Reagent	Volume
10x Reaction buffer	2µl
25mM $MgCl_2$	4µl
Deoxyribonucleotide mix	2µl
Primer oligo dT	2µl
RNAse inhibitor	1µl
AMV reverse transcriptase	0.8µl
Sterile water	7.2µl
RNA	1µl
Total volume	**20µl**

Following cDNA synthesis, real-time PCR was carried out in the LightCycler using SYBR Green chemistry, which is a fluorescent dye that is specific to double-stranded DNA. The forward and reverse primers used were specific for 5' DWNN-13, 3' DWNN-200 and exon 16. The primer sequences were as follows:

5' DWNN-13 Forward: aggaatctgctcttaccttggc; Reverse: cattggtgatctgcaggtcgc

3' DWNN-200 Forward: ctactatgatcagttggatatta; Reverse: tggaaaatgagcgcctac

Exon 16 Forward: caatctcacacttttctacacat; Reverse: ctactatgatcagttggatatta

The reaction was made up in capillaries in precooled centrifuge adapters as follows using the Roche LightCycler FastStart DNA Master SYBR Green I kit:

Table 2.2: Cocktail for RT-PCR

Reagent	Volume
H_2O, PCR grade	11.6µl
$MgCl_2$ stock solution	2.4µl
LightCycler FastStart DNA Master SYBR Green I	2µl
Forward primer	1µl
Reverse primer	1µl
DNA template	2µl
Total	**20µl**

The QT-PCR reaction was set up as follows:

Table 2.3: Reactions for RT-PCR

Programme	Target temperature	Holding time	Acquisition
Activation (1 cycle)	95°C	10 mins	None
PCR (40 cycles)	95°C	10 secs	None
	50°C	10 secs	None
	72°C	15 secs	Single
Melt (1 cycle)	95°C	0 secs	None
	65°C	30 secs	None
	95°C	0 secs	Continuous
Cooling (1 cycle)	40°C	10 secs	None

2.3.1 Control

A negative control was included in the RT-PCR reaction by omitting the DNA template. The reference gene GADPH was used as a positive control.

2.4 *In situ* hybridisation

This involves the hybridisation of a labelled nucleic acid probe directly to cells in a tissue section. This is performed to quantify the levels of DWNN mRNA in the prepared tissue sections. A probe is synthesized which is complementary to a specific transcript of the DWNN gene. Altogether, three such probes were used to ascertain the expression of each transcript in the diseased and control tissues.

2.4.1 Probe preparation

2.4.1.1 Ligation and transformation

The transcript was cloned into a vector, which is the pGEM T-Easy vector because this allows for the cloning of PCR products without purification. Cloning is necessary for sequencing and probe synthesis. This was followed by transformation of competent cells, which involves the vector with the insert being incorporated into a cell and that cell taking on the characteristics of the insert.

1. The trancript was ligated into the pGEM T-Easy vector using the Promega LigaFast kit (Promega, Madison WI, USA) in the following method:

Table 2.4: Cocktail for ligation

Ligation	Reaction	Negative control
DNA product	1µl	-
2x ligation buffer	5µl	5µl
PGEM T-Easy (Promega, Madison WI, USA)	1µl	1µl
T4 ligase (Promega, Madison WI, USA)	1µl	1µl
Sd H_2O	2µl	3µl
Total volume	10µl	10µl

The reaction and negative control were then incubated at room temperature for two hours.

2. *E. coli* MC1061 (Fermentas, Hanover, MD, USA) competent cells were thawed on ice. 100µl of these cells were then added to both the ligations and incubated for 30 minutes on ice.
3. They were immediately incubated in a 37˚C waterbath for five minutes, and then put on ice for two minutes.
4. 900µl of LB, lacking ampicillin, was added and incubated at 37˚C for an hour.

5. 100µl of the reaction and negative control are plated on warmed ampicillin-containing agar plates respectively. These were incubated overnight at 37˚C.
6. After colonies had grown, each colony was suspended in 10µl sd H_2O.
7. From this solution, 5µl was added to 5ml LB containing 5µl ampicillin (Roche, Mannheim, Germany) (this was used for miniprepping). 900µl of this solution was added to 100µl of glycerol (Saarchem Merck, Gauteng, South Africa) and stored at -70˚C as stock.
8. The solution was also used to perform a 3-way streak on agar plates containing ampicillin. This was to confirm that the insert was present.

2.4.1.2 Miniprep (Centrifugation protocol)

This is performed to efficiently and effectively isolate plasmid DNA.

The Promega WizardPlus SV Miniprep DNA Purification System (Promega, Madison WI, USA) was used:

1. The overnight culture was centrifuged for five minutes and the pellet resuspended in 250µl Cell Resuspension Solution. This was resuspended by vortexing.
2. 250µl of Promega Cell Lysis Solution (0.2M NaOH and 1% SDS) was added, and inverted four times to mix. The solution was incubated at room temperature, for about five minutes till it cleared.
3. 10µl of Alkaline Protease Solution (Promega, Madison WI, USA) was added, inverted four times, and incubated for five minutes at room temperature.

4. 350µl of Neutralization Solution was added and inverted four times. This was centrifuged at top speed for ten minutes at room temperature.
5. A spin column was inserted into a collection tube, and the cleared lysate decanted into the spin column.
6. This was centrifuged at about 14 000 x g for one minute at room temperature, the flowthrough discarded and the column reinserted into the collection tube.
7. 750µl of Wash Solution (to which ethanol [Merck, Gauteng, South Africa] has been added) was added and centrifuged at top speed for one minute. The flowthrough was discarded and the column reinserted into the collection tube.
8. 250µl of Wash Solution was added, centrifuged for one minute and the flowthrough discarded. The column was reinserted into the collection tube.
9. The spin column was transferred to a sterile 1.5ml microcentrifuge tube and 100µl of DEPC water (Sigma, Steinheim, Germany) added. This was centrifuged at top speed for one minute at room temperature.
10. The column was discarded and the DNA stored at -20˚C.

2.4.1.3 Restriction digestion

This was to confirm that the correct insert was cloned by ascertaining the size of the insert. PST linearises the vector by cutting at the SP6 promoter region. EcoR1 releases the insert by cutting at the T7 promoter region and the SP6 promoter region, thereby cutting on either side of the insert.

1. The digestion was performed as follows on the DNA prepared above:

Table 2.5: Restriction digestion

EcoR1	PST
10µl clone	10µl clone
1µl 10x buffer H	1ul 10x buffer H
1µl EcoR1	1µl PST
18µl sd H2O	18µl sd H_2O

2. These were incubated for an hour in a 37˚C waterbath.
3. They were then placed in a 65˚C heating block for five minutes and loaded onto a 1% agarose gel.
4. The controls used on the gel were the uncut plasmid and the insert.

2.4.1.4 Isolating band from gel

A 1.5ml microcentrifuge tube was weighed. The agarose gel was viewed under a UV light illuminator (UMP, California, USA) and the appropriate PST cut band excised using

a clean razor blade. This fragment was put into the clean tube and the weight recorded. The weight of the band was then calculated by subtraction.

2.4.1.5 Dissolving the gel slice

The Promega Wizard SV Gel and PCR Clean-up system was used:

1. Membrane Binding Solution was added to the microcentrifuge tube at a ratio of 10µl of solution per 10mg of agarose gel slice.
2. This was vortexed and incubated at 50-65˚C for ten minutes, or until the gel slice had dissolved. The tube was vortexed every few minutes to increase the rate of agarose gel melting. It was then briefly centrifuged at room temperature.

2.4.1.6 DNA purification

1. An SV Minicolumn was placed in a collection tube and the dissolved gel mixture transferred to this assembly. This was incubated for one minute at room temperature.
2. It was then centrifuged in a microcentrifuge at 10 000 x g for one minute and the liquid in the collection tube discarded.
3. 700µl of Membrane Wash Solution (diluted with 95% ethanol) was added to the SV Minicolumn and centrifuged for one minute at 10 000 x g.
4. The collection tube was emptied and 500µl of Membrane Wash Solution added. This was centrifuged for five minutes at 10 000 x g.
5. The Minicolumn was transferred to a clean 1.5ml microcentrifuge tube and 50µl of DEPC water added. This was centrifuged for one minute at 10 000 x g.

6. The tube was stored at -20˚C.

2.4.1.7 Spectrophotometry

1. The purified DNA was quantified by spectrophotometry. Readings were taken at 260nm and 280nm and the DNA concentration calculated.
2. As a DNA concentration of 2µg/µl is needed for Dig labelling, the amount of DNA that must be added was calculated from the concentration.

2.4.1.8 Digoxigenin labelling

1. The clone was cut with PST to linearise the vector. As a DNA concentration of 2µg/ml is needed for labelling, the DNA was diluted.
2. The following methods were applied:

Table 2.6: Labelling with Digoxigenin

Reagents	Antisense cRNA to DWNN	Sense cRNA to DWNN	Control cRNA
DWNN cDNA digested with ApaI	-	2µl (1µg)	
DWNN cDNA linearised with PST	10.5µl (1µg)	-	
Control DNA	-	-	8µl (1µg)
10x NTP labelling mix	2µl	2µl	2µl
10x Transcription buffer	2µl	2µl	2µl
DEPC water	3.5µl	12µl	6µl
SP6 RNA polymerase (2U/µl)			2µl
T3 polymerase (2U/µl)	-	2µl	
T7 polymerase (2U/µl)	2µl	-	
Total	20µl	20µl	20µl

3. They were incubated in a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems, Singapore) for two hours at 37˚C.
4. To stop the reaction, 2µl of 0.2M EDTA (pH 8.0) (Saarchem Merck, Gauteng, South Africa) was added, mixed and briefly centrifuged.
5. 4.4µl of 4M LiCl (Sigma, Steinheim, Germany) was added to each reaction and 150µl of cold absolute ethanol to precipitate the reactions.
6. After mixing and a quick centrifugation, the reactions were incubated at -20°C for two hours or overnight.
7. The pellet was precipitated by centrifuging for 15 minutes at 13 000 x g at 4°C. The supernatant was decanted.
8. The pellet was washed with 200µl of cold 70% ethanol. The pellet was centrifuged for 15 minutes at 13 000 x g at 4°C. The supernatant was removed using a pipette.
9. The pellet was dried for two hours in a laminar flow and then dissolved using 50µl of DEPC-treated water.
10. The dissolved pellet was left at 4°C for about an hour. The Dig labelled cRNA was stored in 2 x 20µl and 1 x 10µl aliquots at -70°C.

2.4.1.9 Estimation of minimal probe concentration

Table 2.7: Dilutions of labelled probes

Dilution	Dig labelled control RNA (BM)	Dig labelled cRNA to control DNA	Sample probe (AS and S)
Initial concentration	10µg/100µl	250ηg/50µl	10µg/50µl
Dilution 1 [20ηg/µl]	1:5 1µl + 4µl dH_20		1:10 1µl + 9µl dH_20
Dil. 2 [1ηg/µl]	1:20 2µl + 38µl dH_20	1:5 2µl + 8µl dH_20	1:20 2µl + 38µl dH_20
Dil. 3 (1:10) [100ρg/µl]	5µl + 45µl dH_20	5µl + 45µl dH_20	5µl + 45µl dH_20
Dil. 4 (1:10) [10ρg/µl]	5µl + 45µl dH_20	5µl + 45µl dH_20	5µl + 45µl dH_20
Dil. 5 (1:10) [1ρg/µl]	5µl + 45µl dH_20	5µl + 45µl dH_20	5µl + 45µl dH_20
Dil. 6 [0.1ρg/µl]	5µl + 45µl dH_20	5µl + 45µl dH_20	5µl + 45µl dH_20
Dil. 7 [0.01ρg/µl]	5µl + 45µl dH_20	5µl + 45µl dH_20	5µl + 45µl dH_20

2.4.1.10 Concentration estimation for the probe

The optimal concentration of the antisense and sense probes was determined by comparing the spot intensities to Dig labelled control DNA.

1. Using a pencil, spots were lightly marked on a Nylon membrane (Hybond, Amersham Biosciences, California, USA).
2. 1µl of each dilution was spotted onto the membrane and allowed to air dry for 10 minutes.
3. The probe was fixed to the membrane under UV light for five minutes.
4. 1 x washing buffer was added to cover the membrane while shaking for five minutes.
5. 1 x blocking buffer was added to cover the membrane while shaking for 30 minutes.
6. Anti-Dig alkaline phosphatase was diluted 1:10 000 with 1 x blocking buffer and added to cover the membrane. This was incubated for 30 minutes.
7. 1 x washing buffer was added to cover the membrane while shaking for 15 minutes. This wash was repeated.
8. The membrane was covered with 1 x Detection buffer and shaken for two minutes.
9. NBT/BCIP (Roche, Mannheim, Germany) was diluted 1:50 with detection buffer and added to immerse the membrane. This was incubated overnight to allow the colour to develop.
10. The colour reaction was terminated by adding 50ml of TE buffer for five minutes.
11. The membrane was air-dried and the minimal dilution of the probe that could be detected compared to the Dig labelled control RNA is estimated.

2.4.2 *In situ* hybridisation

2.4.2.1 Pre-hybridisation

1. Paraffin wax sections were dewaxed in three changes of xylene for ten minutes each.
2. They were rehydrated in two changes of absolute ethanol for three minutes each. They were further rehydrated in 90%, 70%, 50% ethanol and DEPC water for three minutes each.
3. The sections were fixed in freshly prepared 4% PFA for 20 minutes at room temperature. Following this, they were rinsed in three changes of TBS for one minute each.
4. The sections were denatured in 0.1M HCl for 10 minutes. They were then rinsed in three changes of TBS for one minute each.
5. Slides were immersed in 0.5% acetic anhydride in 100mM Tris for ten minutes. They were rinsed in three changes of TBS for one minute each.
6. 100µl of proteinase K (Promega, Madison WI, USA) (2µl in 998µl TBS) was added to each slide and incubated for 20 minutes at 37˚C. The slides were rinsed in three changes of TBS for one minute each.
7. The slides were incubated in TBS for five minutes at 4°C.
8. The sections were dehydrated in 50%, 70% and 90% ethanol for one minute each. They were dehydrated in two changes of absolute ethanol for one minute each.
9. The slides were dried in chloroform for ten minutes in the fume hood.

2.4.2.2 Hybridisation

1. The probes were thawed on ice.
2. 12µl of Herring Sperm DNA (HSD 10µg/ml) (Promega, Madison WI, USA) was added to 1188µl of hybridisation buffer. 1µl of the probe was added to this solution and put in rapidly boiling water for five minutes, and then cooled on ice.
3. 100µl of each probe preparation was spread on a suitable section limited by an area enclosed by a gene frame (Southern Cross Biotechnology, Cape Town, South Africa).
4. A solution of 5x SSC buffer and 50% formamide was poured into the hybridisation chamber.
5. Hybridisation was carried out in a Hybaid Omnislide Flat Block Humidity Chamber (Thermo Electron Company, Massachusetts, USA) at 55°C for 16 hours.

2.4.2.3 Post-hybridisation and detection

The Roche Diagnostics Washing and Blocking Kit (Roche, Mannheim, Germany) was used:

1. The slides were removed from the humid chamber, excess probe dropped off and the gene frames removed.
2. The slides were washed in 2x SSC for 30 minutes at 37°C in a hybridisation oven. They were then washed in 2x SSC, 1x SSC and 0.1x SSC for 20 minutes each at 55°C.
3. The slides were rinsed in three changes of TBS for one minute each at room temperature.

4. 100µl of 1x blocking buffer was added to each slide and they were incubated in a humidity chamber for 15 minutes at room temperature.

2.4.2.4 Colorimetric ISH

1. The slides were incubated with 100µl of anti-Dig IgG, conjugated with alkaline phosphatase diluted 1:500 in 1x blocking solution, in a humidity chamber for one hour at room temperature.
2. They were rinsed in three changes of TBS for one minute each.
3. They were incubated with 100µl of Chromogen NBT/BCIP diluted 1:50 in 1x detection buffer. This was allowed to develop in a dark humid chamber overnight at room temperature.
4. Slides were placed in 1x TE for five minutes to stop the reaction. They were rinsed in running tap water for five minutes.
5. Mayer's Haematoxylin was added for 15 minutes to counterstain. They were rinsed in running tap water for ten minutes.
6. The slides were mounted with permanent aqueous glycerol medium and allowed to dry. They were viewed under a light microscope and the images captured by using a Zeiss Microscope camera and the Axio software programme.

2.4.2.5 Fluorimetric ISH

1. The slides were washed in TBS-Tween for five minutes.
2. They were incubated in TNB for 30 minutes at 37ºC.
3. The slides were incubated with 75µl of Anti-Dig-fluorescein with TNB (diluted 1:500) for 30 minutes at 37ºC.
4. This was followed by three washes in TBS-Tween for five minutes each.
5. The sections were mounted using the Molecular Probes SlowFade® Light Antifade Kit (Oregon, USA):

5.1. One drop of component C (equilibration buffer) was added per section.

5.2. After ten minutes at room temperature, one drop of component A (antifade reagent in glycerol buffer) was added to a coverslip and put on top of the slide.

6. The sections were kept in the dark and viewed under the Zeiss fluorescence microscope.

2.4.2.6 Controls

Testes tissue was the positive control, as high levels of DWNN have been demonstrated here previously and it is to this standard that the experimental tissues are compared to in order to determine if labelling has occurred. The negative control was run by omitting the addition of a probe. These controls allow one to differentiate between true staining and background staining. A third control was the use of normal colon tissue to compare levels of expression between undiseased and diseased tissues.

2.5 Immunocytochemistry (DWNN)

This was done to determine the localization of DWNN proteins. It involves using Dig-labelled (Roche, Indiana, USA) antibodies (antihuman DWNN antibody) to localize specific antigens found on the surface of proteins (DWNN). The DAKO LSAB+ Kit Peroxidase was used:

1. Tissue sections were dewaxed in two changes of xylene for ten minutes each at room temperature.
2. Sections were rehydrated in two changes of absolute ethanol for five minutes each.
3. The sections were immersed in 100% methanol for 20 minutes.
4. They were rehydrated in two changes of 95% ethanol for four minutes each. They were rehydrated in 70% ethanol for three minutes and washed in distilled water for five minutes.
5. The sections were heated in 0.1M sodium citrate (pH 6.0) in a microwave and held at 80°C for two minutes. This was allowed to cool to room temperature for 20 minutes.
6. They were washed in PBS-1% BSA for two minutes.
7. 100µl of 3% Hydrogen peroxide was added to each slide and left to develop for 30 minutes.
8. 100µl of 1x Blocking buffer was added to each slide and left for an hour.
9. The primary antibody (Anti-DWNN) was thawed in ice, spun at maximum speed and 1µl of the supernatant was added to 1000µl of PBS-BSA.

10. 100µl of anti-DWNN preparation was added to each slide and incubated overnight at 4°C in a humidity chamber.
11. The slides were washed with PBS-BSA for two minutes.
12. 100µl of Antibody Linker was added to each slide and incubated for 30 minutes at room temperature. They were washed with PBS-BSA for two minutes.
13. 100µl of prepared DAB chromogen and substrate buffer solution was added to each section and allowed to develop to a light brown colour.
14. Mayer's Haemotoxylin is added for ten minutes and the sections were washed under running tap water for five minutes.
15. They were dehydrated in 70%, 95% and 100% ethanol for one minute each. They were dehydrated in xylene for one minute.
16. The slides were mounted with xylene-based mounting media, dried and viewed under a light microscope. Images were captured with a Zeiss camera using the Axio software programme.

2.5.1 Controls

A negative control was included by not adding the anti-DWNN. Normal colon tissue was also used to compare differences in expression in diseased tissue.

2.5.2 Statistical analysis

1. Images of normal and cancerous tissues stained with DAB were imported into an analysis software package.

2. The areas of interest were divided into 3-4 regions to obtain a higher n value. The area of each region was calculated in μm^2.
3. As DAB immunolabeling falls between 160 and 256 pixels, the total number of pixels that fall within this area was calculated in each region (pixels/ μm^2).
4. The average values for each image were calculated across all available data points. This value was multiplied by a hundred to obtain a more comprehensible value.

2.6 Immunocytochemistry (*Helicobacter pylori*)

The purpose of this was to localise the *H. pylori* protein in normal and cancerous colon sections to study the correlation between bacterial occurrence and colon cancer incidence. A peroxidase labelled antibody to *H. pylori* (0.1mg/ml) (Kirkegaarde and Perry Laboratories, Maryland, USA) was employed. This antibody recognizes the following serotypes from the American Type Culture collection: *H. pylori* 43504, *H. pylori* 43526 and *H. pylori* 43579. The methodology is the same as for 2.5 Immunocytochemisty (DWNN), and the antibody was diluted 1:100 in PBS.

2.6.1 Controls

A negative control was obtained by omitting the antibody from the reaction. Normal colon tissue was also included in the experiment to examine localisation in undiseased cases.

2.7 TUNEL

Apoptosis results in a large number of DNA strand breaks and these can be detected by labelling the 3' OH termini in DNA breaks with biotin. This reaction is catalysed by exogenous terminal deoxynucleotide transferase (Tdt). Streptavidin labelled with horseradish-peroxidase (HRP) is bound to these nucleotides. In the presence of diaminobenzidine (DAB), apoptotic nuclei are stained dark brown.

The Promega DeadEnd™ Colorimetric TUNEL system kit (Promega, Madison WI, USA) was utilised:

1. Sections were dewaxed in two changes of xylene for ten minutes each. They were washed in two changes of absolute ethanol for five minutes each and immersed in methanol for 20 minutes.
2. They were rehydrated in 95%, 85%, 70% and 50% ethanol for three minutes each.
3. The sections were washed in 0.85% sodium chloride for five minutes.
4. They were then washed in 1% PBS for five minutes and fixed in 4% PFA for 15 minutes.
5. The slides were washed in three changes of PBS for three minutes each.
6. 100µl of proteinase K (20µg/µl) diluted 1:500 with PBS, was added to each section and incubated for 20 minutes. This was washed off by immersing the slides in PBS for five minutes.
7. The slides were immersed in 4% PFA for five minutes and then washed in two changes of PBS for five minutes each.
8. 100µl of equilibration buffer was added to each slide for ten minutes.

9. 98µl of equilibration buffer was added to 1µl of bionylated mix and 1µl of Tdt enzyme. 100µl of this was added to each slide and incubated for an hour in a humidity chamber at 37°C.
10. The slides were immersed in 2x SSC for 15 minutes. The sections were washed in three changes of PBS for five minutes each.
11. 100µl of 0.3% hydrogen peroxide was added to each slide for five minutes. They were washed in three changes of PBS for five minutes each.
12. 100µl of streptavidin HRP, diluted 1:500 in PBS, was added to each section and incubated for 30 minutes.
13. The slides were immersed in three changes of PBS for five minutes each.
14. 100µl of the prepared DAB solution was added to each slide and incubated until a light brown colour developed.
15. The slides were rinsed in three changes of deionised water. They were dehydrated in 50%, 70%, 85% and 95% ethanol for one minute each.
16. They were dehydrated in absolute ethanol for one minute, repeated once, and immersed in xylene for one minute.
17. The slides were mounted in xylene-based Entellan and viewed under a light microscope. The Zeiss camera and Axio software programme were used to capture images.

2.7.1 Controls

A negative control is included by not adding TdT enzyme. Normal colon tissue was also included in these reactions to compare apoptosis occurrence compared to diseased tissue.

2.8 Proliferation assay

Ki-67 is a proliferation marker and by using antibodies against it, the quantity of cells that are dividing can be visualized. The Ki-67 goat polyclonal immunoglobulin-G (200µg/ml) was used in conjunction with a biotinylated donkey anti-goat immunoglobulin-G antibody and a staining system to produce a colorimetric reaction.

The Santa Cruz Biotechnology ABC Staining System (California, USA) was used:

1. The slides were deparaffinized in three changes of xylene, of five minutes each.
2. They were immersed in two changes of 100% ethanol, of ten minutes each.
3. This was followed by two changes of 95% ethanol, of ten minutes each.
4. The sections are placed in water for two minutes, with stirring.
5. They were covered with 10mM sodium citrate (pH 6) and heated at 95ºC for five minutes.
6. Fresh buffer was added, and heated for a further five minutes at 95ºC. They were then left to cool for 20 minutes.
7. The sections were washed in three changes of distilled water, at two minutes each.
8. 1.5% blocking serum in PBS was added to the sections and left to incubate for one hour.
9. The primary antibody diluted in PBS to a final concentration of 3µg/ml with 1.5% normal blocking serum (1.5µl in 98.5µl) was added to each section and left for 30 minutes at room temperature.
10. The sections were washed in PBS for three changes of five minutes each.

11. The biotin-conjugated secondary antibody (1µg/ml) diluted in PBS with 1.5% normal blocking serum (1µl antibody in 99µl blocking serum) was added for 30 minutes.
12. This was followed by three washes in PBS of five minutes each.
13. Avidin biotin enzyme reagent was added for 30 minutes.
14. This was again followed by three washes in PBS of five minutes each.
15. The peroxidase substrate was added till the desired intensity had developed, ranging from between thirty seconds to ten minutes.
16. The sections were washed in water for five minutes.
17. They were immersed in 95% ethanol for two changes of ten seconds each.
18. They were then put into two changes of 100% ethanol for ten seconds each.
19. The slides were put into three changes of xylene, of ten seconds each.
20. The sections were mounted with entellan and a coverslip and viewed under the Zeiss light microscope.

2.8.1 Controls

Normal colon tissue was also used in the experiment to compare proliferation between undiseased and diseased cases.

2.9 Bcl-2 assay

Bcl-2 is an inhibitor of apoptosis. The purpose of this experiment was to visualize where in the tissues this protein was found and to examine any correlation with its localisation and DWNN localisation and from this draw conclusions about the role of DWNN in apoptosis. The Bcl-2 mouse anti-human monoclonal immunoglobulin-G (200μg/ml) (Santa Cruz Biotechnology, California, USA) was used in conjunction with a biotinylated goat anti-mouse immunoglobulin-G antibody and a biotinylated staining system, which resulted in a colorimetric reaction.

The Santa Cruz Biotechnology ABC Staining System was used and the methodology is the same as for 2.8 Proliferation assay.

2.9.1 Controls

Bcl-2 localisation in normal colon tissue was also examined to compare this to localisation in cancerous colon tissue.