CHAPTER THREE: RESULTS

3.1. Histopathology

3.1.1 Introduction

The histology of normal colon tissue and the histopathology of colon cancer tissue had to be elucidated before experimental results could be analysed. Mayer's haematoxylin was used in conjunction with the eosin counterstain as it clearly identifies nuclei after bluing in running water, while eosin stains the cytoplasm various shades of pink, thereby allowing for differentiation of the various tissues (Luna, 1960).

3.1.2 Normal colon tissue

The normal colon tissue is composed of the epithelium, lamina propria, muscularis mucosae (which altogether constitute the mucosa), submucosa, muscularis propria, subserosa and serosa. A single layer of absorptive and goblet columnar cells lines the surface and the crypts of Lieberkühn (**figure 3.1**). The tubules are evenly arranged parallel to each other and contain endocrine, regenerative and Paneth cells, which are thought to be secreted antimicrobial elements. Lymphoid-glandular complexes are part of the mucosa associated lymphoid tissue and can be found scattered randomly in the mucosa, often protruding into the submucosa (Owen and Kelly, 1994; Levine and Haggitt, 1997).

The lamina propria is composed of connective tissue occurring between the surface epithelium and the muscularis mucosae, containing plasma cells, lymphocytes (mostly T-cell), macrophages and eosinophils. The muscularis mucosa is a sheet of smooth muscle cells separating the lamina propria from the submucosa. The submucosal

layer is composed of loose connective tissue with blood vessels and nerves. The muscularis propria has an inner circular layer and an outer longitudinal layer of muscle. The subserosa is composed of connective tissue occurring in between the muscularis propria and the serosa, which is a mesothelial layer (Owen and Kelly, 1994; Levine and Haggitt, 1997).

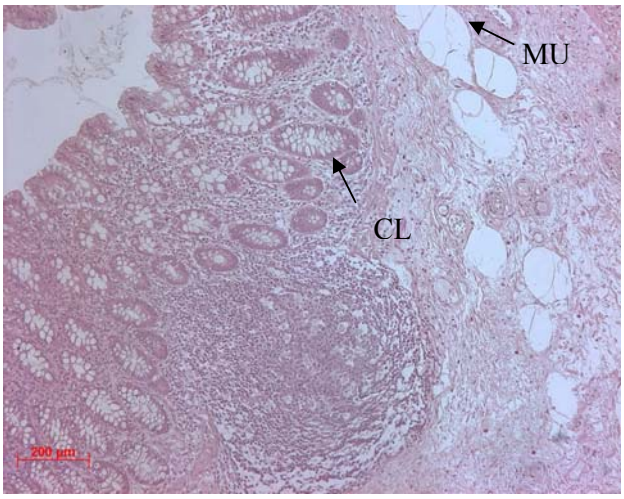


Figure 3.1: Normal colonic tissue showing the crypts of Lieberkühn (CL) in the lamina propria and mucin pools (MU) (x100).

3.1.3 Tumour classification

Tumours are classified into levels of differentiation based on the percentage of dysplastic tissue that form glands compared to those that do not form glands. The majority of tumours in the colon can be classified as well to moderately differentiated, consisting of irregular glands lined with tall columnar or cuboidal epithelium, often with necrotic debris in the lumina. The World Health Organization (WHO) grades adenocarcinomas as follows:

Grade X – grade cannot be established

Grade 1 – well differentiated where more than 95% of the tumour has formed glands (**figure 3.4**)

Grade 2 – moderately differentiated where 50-95% of the tumour has glandular formation (**figure 3.3**)

Grade 3 – poorly differentiated where 5-49% of the tumour is composed of glands

Grade 4 – undifferentiated where less than 5% of the tumour has formed glands (Hamilton and Aaltonen, 2000; **figure 3.2**).

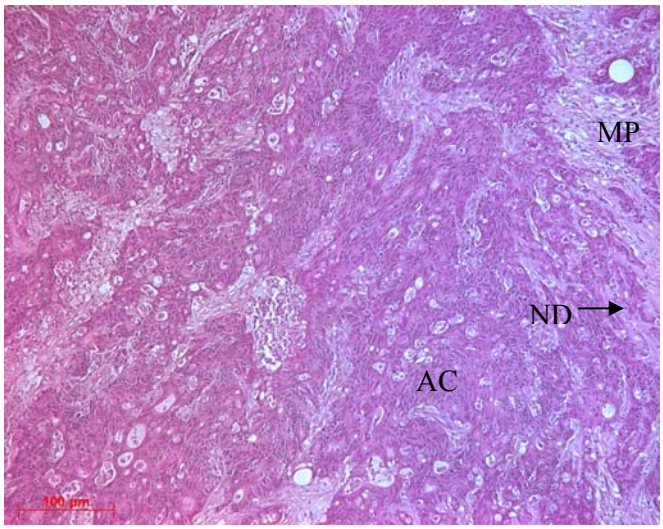


Figure 3.2: Poorly differentiated adenocarcinoma (AC) as characterised by lack of glandular formation. Mucin pools (MP) occur scattered throughout the diseased tissue as well as areas of necrotic debris (ND) (x100).

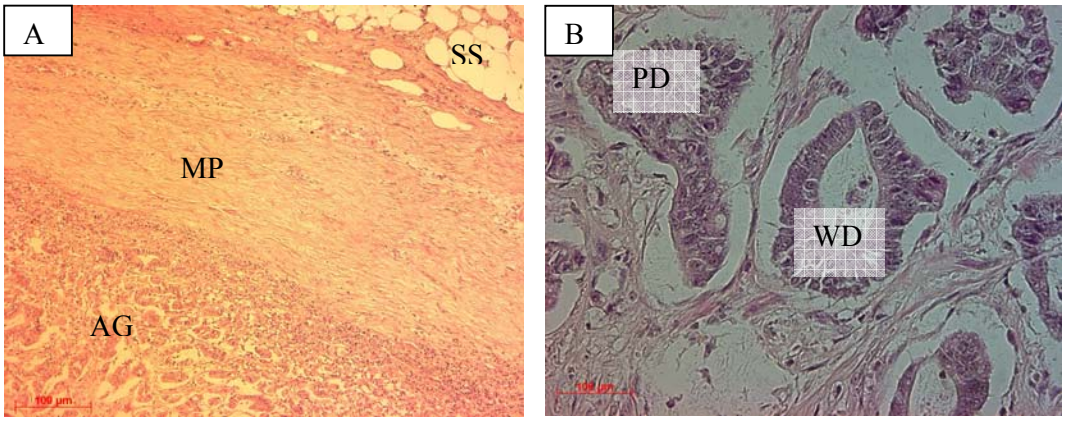


Figure 3.3: Moderately differentiated adenomatous glands (AG) in muscularis propria (MP) adjacent to the subserosa (SS). This level of differentiation is characterised by intermediate formation of well differentiated (WD) and poorly differentiated (PD) glandular structure (A=x100; B=x400).

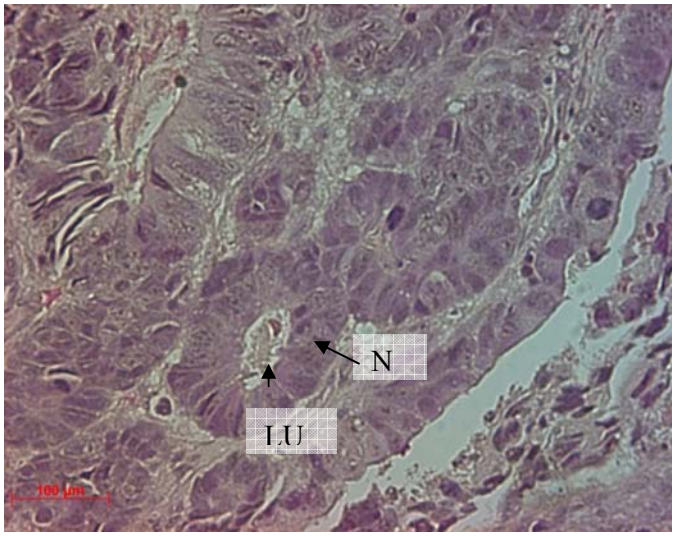


Figure 3.4: Well differentiated adenocarcinoma characterised by good glandular structure. This is typified by lumen (LU) formation; the epithelium shows crowded cells as seen by dense nuclei (N) and this is typical of cancer (x400).

An adenoma by definition is a benign epithelial neoplasm with tubular and/or villous architecture, which exhibits dysplasia both structurally and cytologically. All adenomas have at least low grade dysplasia. In neoplastic progression an adenoma showing high grade dysplasia or adenocarcinoma *in situ*, where the basement membrane is still intact, advances into an adenoma with intramucosal carcinoma, where there is invasion of the lamina propria. When the submucosa is invaded, the carcinoma has advanced to adenoma with invasive carcinoma (**figure 3.5**), and beyond this stage the carcinoma is classified as metastatic (**figure 3.6**) (Cooper *et al.*, 1998).

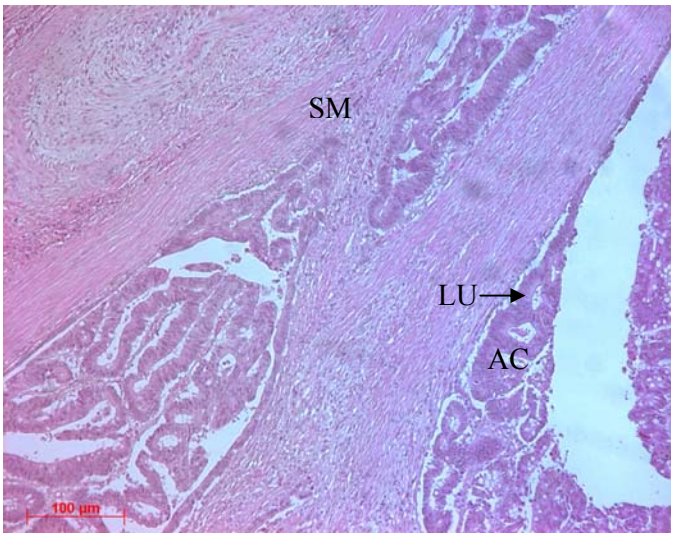


Figure 3.5: Well differentiated adenocarcinoma (AC) in the smooth muscle (SM) of the submucosa. The formation of glands as seen by lumen (LU) formation characterises this level of differentiation (x100).

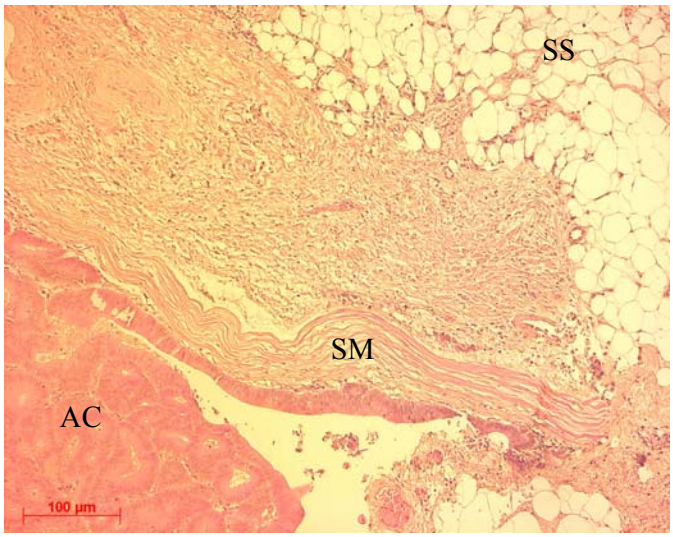


Figure 3.6: Well differentiated adenocarcinoma (AC) in smooth muscle (SM) in muscularis propria adjacent to the fat cells of the subserosa (SS) (x100). As the dysplastic glands have penetrated beyond the submucosa, it is now classified as an invasive carcinoma.

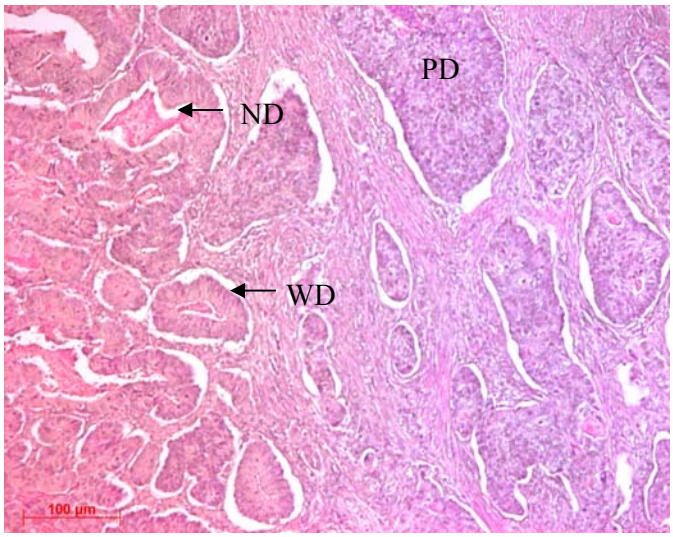


Figure 3.7: Moderately differentiated adenomatous glands in the submucosa typified by both poorly (PD) and well differentiated (WD) structures (x100). Necrotic debris (ND) can be seen in the lumens of the glandular structures, indicative of apoptosis occurring in the vicinity.

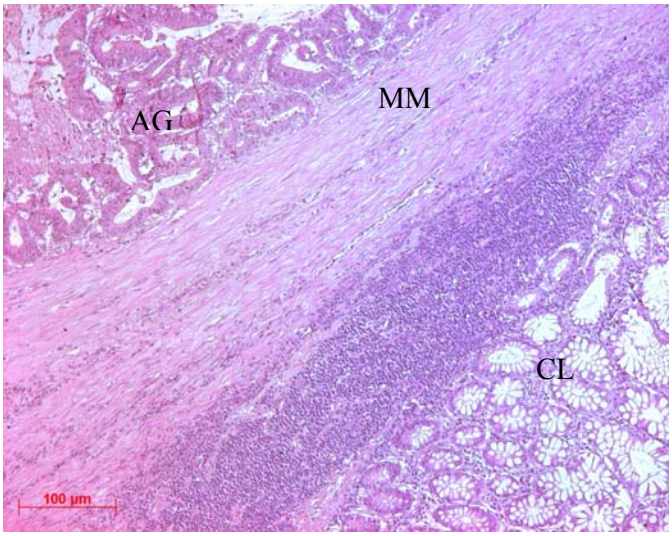


Figure 3.8: Well differentiated adenomatous glands (AG) in the submucosa adjacent to the muscularis mucosa (MM) and crypts of Lieberkühn (CL) (x100). The dysplastic glands are not classified as invasive as they have not penetrated beyond the submucosal layer.

3.1.4 Summary

This histopathology section formed the essential background needed to familiarise oneself with the normal and cancerous colon tissues, particularly as colon cancer occurs at different levels of differentiation. It allowed for the identification of the different tissue layers, the main cell types, normal and dysplastic structures and progressive changes with advancing cancer stages. The haematoxylin and eosin stained sections allowed for changes to be studied and will prove invaluable in further sections where localisation studies are performed.

3.2 Quantitative PCR

The aim of this experiment was to quantify the three DWNN transcripts in DNA extracted from a cancerous colon cell line (HT29) and compare the levels to those present in the DNA extracted from a normal kidney cell line (Graham 293). This would allow for conclusions to be drawn as to what degree the DWNN transcripts are expressed in cancerous cells. Forward and reverse primers for 5' DWNN-13, 3' DWNN-200 and exon 16 were employed. The LightCycler measures the fluorescent signals produced during the log-linear or exponential increase phase of the PCR reaction. Real-time PCR is both fast and accurate, and the LightCycler eliminates the need for additional purification or analysis, and reduces the risk of contamination as the optical tubes and reaction plates are closed during the process. The SYBR Green fluorescent dye is used which is specific only for double-stranded DNA therefore only PCR product is fluoresced. As the number of PCR cycles increase, the more double-stranded PCR product formed and the higher the fluorescence. The reference gene GADPH was used as a positive control for both cell-lines as it is constitutively expressed in all cells.

Results showed that firstly, the GADPH gene was found at the highest levels in both the normal and cancerous cell-lines (**figure 3.9**). The 5' DWNN-13, which is the full-length DWNN gene, was shown to be present at the highest concentration. This is to be expected as all three transcripts would be synthesized. The gene showed a clear upregulation in colon cancer cells compared to the normal undiseased cells, and this finding is confirmed in the successive experiments. The next highest level of amplification was seen with the exon 16 DNA in the colon cancer cells, followed by the RBBP6 region. This is contradictory as the RBBP6 primers amplify the length of

DNA, which includes the exon 16, therefore RBBP6 should be found at higher concentrations than exon 16. However, in normal cells RBBP6 was found at higher concentrations than exon 16 (**figure 3.9**).

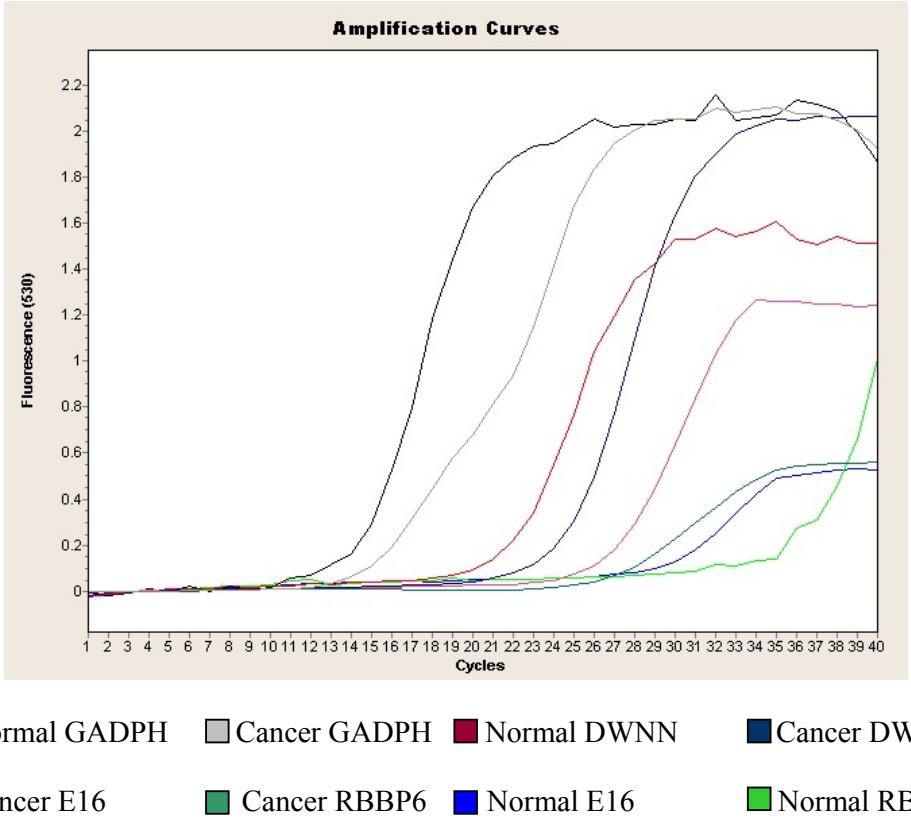


Figure 3.9: Amplification of the DWNN transcripts via real-time PCR in a normal kidney cell-line and a colon cancer cell-line.

3.2.1 Summary

Quantitative PCR showed that the DWNN gene, 3' DWNN 200 and exon 16 were all upregulated in colon cancer cells compared to normal undiseased cells. This suggests that the DWNN transcripts must play a role in cancer progression and the following experiments aim to elucidate if this role promotes or hinders the disease.

3.3 *In situ* hybridisation

As there are three transcripts of the DWNN gene, it has yet to be determined whether all three forms are equally involved in apoptosis and expressed at similar levels, or if there is a particular transcript/s that is the key player/s in apoptosis. This was ascertained by colorimetric and fluorescent *in situ* hybridisation (ISH, FISH), which employs a labelled DNA probe which binds to complementary mRNA and localisation is detected under light and fluorescent microscopy.

3.3.1 Probe synthesis of the 1.1kb transcript

3.3.1.1 Ligation and transformation

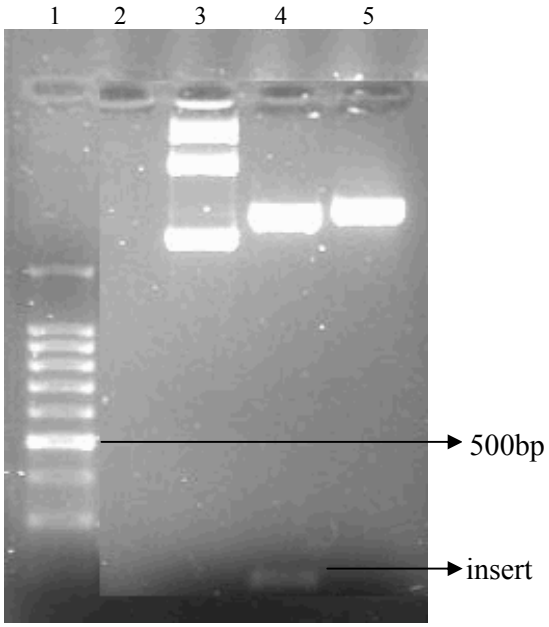
The p21C4 DNA clone complementary to the 3' end of the 1.1kb DWNN mRNA was ligated into the pGEM T-Easy vector using the Promega Ligafast kit. This vector system is used as it has a multiple cloning site allowing for the EcoR1 enzyme to cut at two sites, the SP6 promoter region and the T7 promoter region, thereby releasing the insert from the vector. The PST enzyme was used to linearise the vector as it only cuts at SP6 promoter region. It is also convenient because it has 3'-thymidine overhangs, which allows for correct ligation of the PCR product. With the action of *Taq* polymerase, the PCR product had an adenosine base added to the 3' ends.

E. coli MC1061 competent cells were transformed with the vector containing the insert, incubated with the nutrient-rich Luria broth to promote growth and plated on Ampicillin agar plates. As the pGEM T-Easy vector has an ampicillin-resistant domain, the only colonies that were able to grow on the plates were the cells that had taken up the vector. The positive colonies were able to grow again on new ampicillin agar plates; this was done to confirm the results from the first plates. The positive

colonies were grown overnight in Luria broth containing ampicillin to increase the concentration of colonies for mini-prepping.

3.3.1.2 DNA isolation & linearisation

Mini-prepping was performed to isolate the pGEM T-Easy vectors containing the DNA inserts from the competent cells into which it had been ligated. This was accomplished by using the Promega WizardPlus SV Miniprep DNA Purification System kit. This step was necessary to attain a purer form of the insert of interest. At this point the insert still had to be linearised. This was done by using the PST enzyme. The vector was also cut with the EcoR1 enzyme to establish if the insert was present; this would be clarified by running the two digests on an agarose gel together with the uncut clone from the mini-prep and a DNA marker.



Lane 1: DNA ladder

Lane 3: uncut clone

Lane 4: clone cut with EcoR1 with released 13kDa insert

Lane 5: clone cut with PST

Figure 3.10: Agarose gel showing restriction digestion

The PST band is isolated from the gel and the DNA purified. Spectrophotometric readings are taken to ascertain the purity and concentration of the DNA before labelling. The calculation of the purified linearised clone was calculated at 260µg/ml.

3.3.1.3 Digoxigenin labelling of probe

The Digoxigenin system results in the synthesis of an RNA probe, which is complementary to the 1.1kb transcript, isolated in the steps above. The probe is in the 5' to 3' orientation limiting it to only bind to the 3' end of the complementary mRNA. The Digoxigenin system results in a colorimetric reaction. The labelled probe hydrogen bonds to complementary mRNA. Anti-Digoxigenin binds to the Digoxigenin on the probe. Alkaline phosphatase in turn binds to anti-Digoxigenin and upon addition of the substrate NBT/BCIP a purple/blue colour change occurs as alkaline phosphatase cleaves the substrate. The more copies of DWNN there are, the deeper the shade of blue therefore it is a quantitative technique.

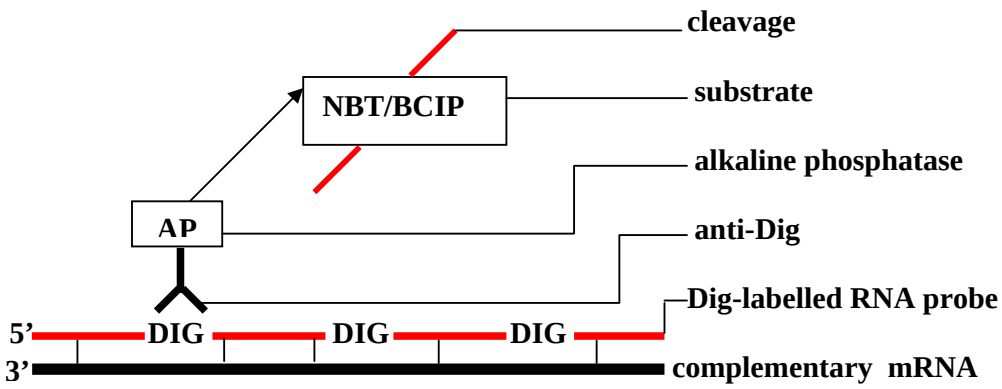


Figure 3.11: Diagrammatic representation of *in situ* hybridisation

Dilutions of the labelled probe were run with a labelled control to ascertain the minimal concentration at which the probe could be detected. Using 14µl of the PST-cut clone of the 1.1kb mRNA, labelling with Digoxigenin showed that this concentration was 10ρg/µl. For *in situ* hybridisation therefore, the probe was diluted to this concentration.

3.3.1.4 Colorimetric ISH images

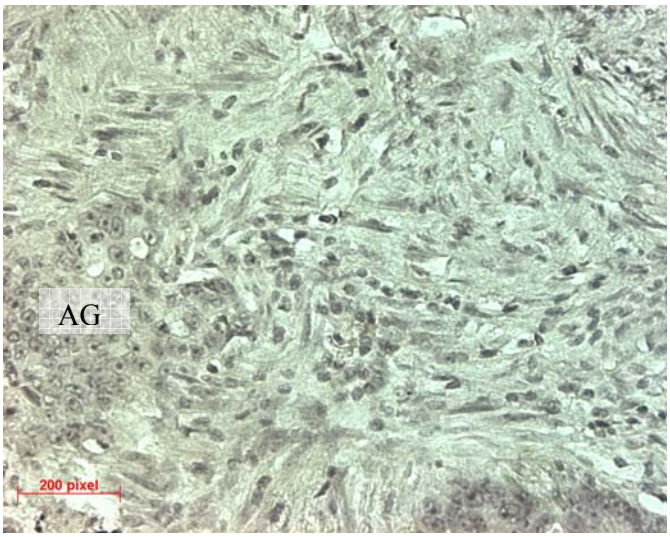


Figure 3.12: Negative control of colon cancer tissue showing moderately differentiated adenomatous glands (AG) in the submucosa as denoted by nuclear and cytoplasmic counterstaining (x400).

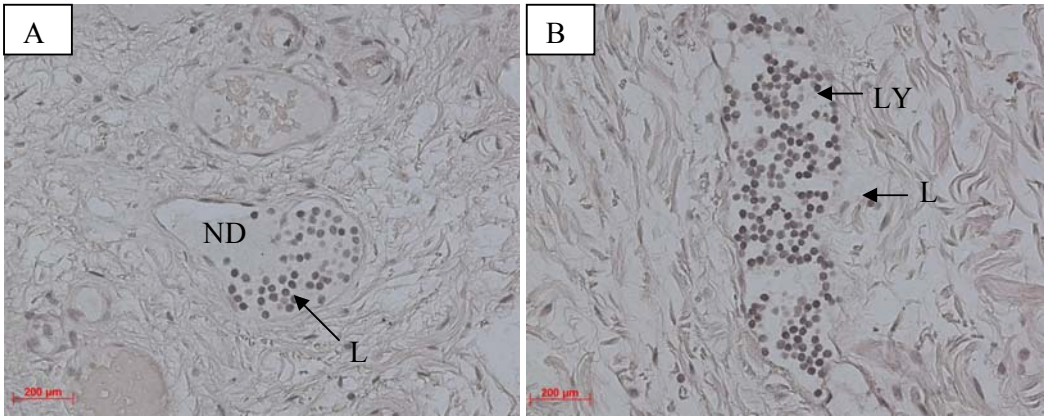


Figure 3.13: (A) Nuclear and cytoplasmic localisation (L) of the 1.1kb DWNN mRNA seen in normal colon tissue in necrotic debris (ND) in the lamina propria (x400); (B) Nuclear and cytoplasmic localisation in lymphocytes (LY) in the lamina propria in normal tissue (x400). Localisation was not seen in other structures, such as the crypts.

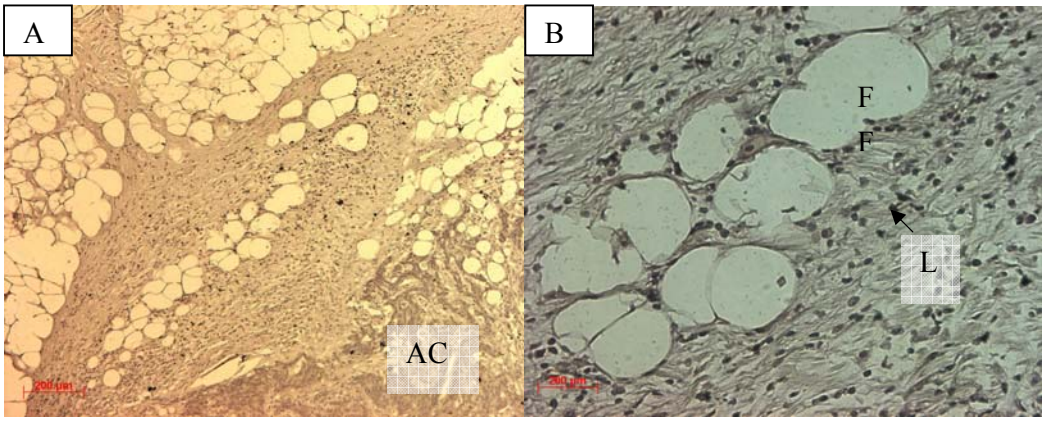


Figure 3.14: Localisation (L) of the 1.1kb mRNA in the subserosa (identifiable by fat cells - F) adjacent to poorly differentiated adenocarcinoma (AC) suggesting a relationship between the DWNN transcript and the carcinomatous tissue. Only by looking at the results from apoptosis detection can it be established whether the transcript aids or hinders cancer progression (A= x100; B= x400).

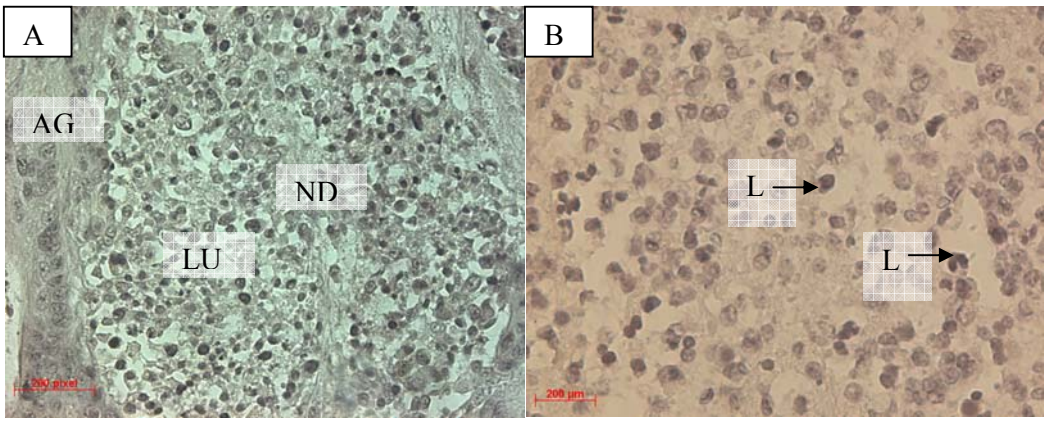


Figure 3.15: Localisation (L) of the 1.1kb mRNA in lymphocytes in necrotic debris (ND) in lumen (LU) of moderately differentiated adenomatous glands (AG) in submucosa suggesting that the DWNN transcript is involved in cell death (A = x400; B = x1000). At higher magnification, it can be seen that the localisation is nuclear (B).

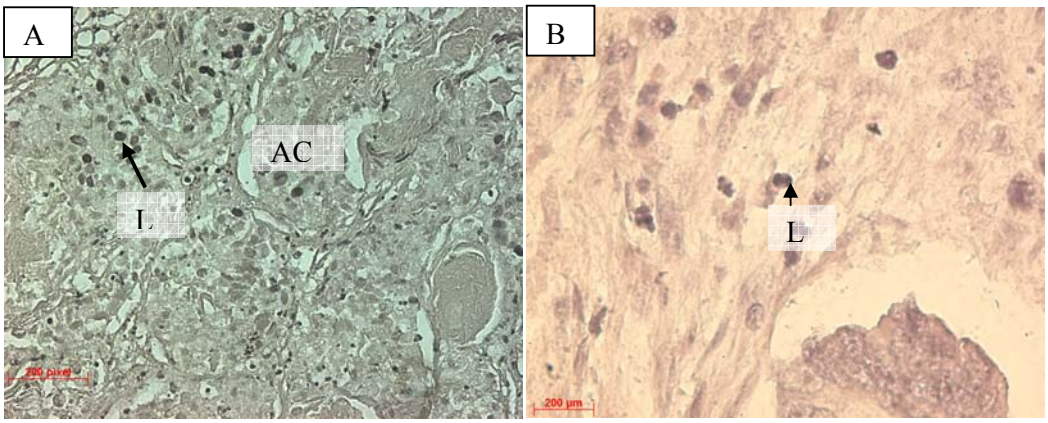


Figure 3.16: Localisation (L) of the 1.1kb mRNA in moderately differentiated adenocarcinoma (AC) in subserosa, again implying a relationship between the gene and carcinomatous cells (A= x400; B= x1000). Higher magnification shows this localisation to be nuclear (B).

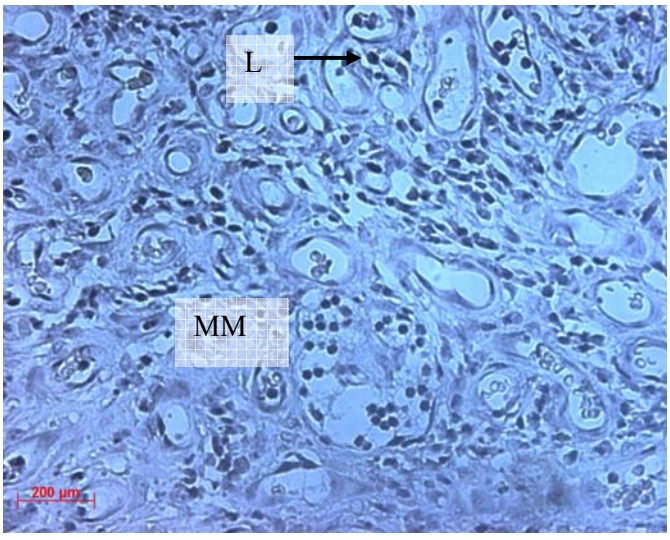


Figure 3.17: Localisation (L) of the 1.1kb DWNN mRNA in muscularis mucosae (MM) adjacent to well differentiated adenomatous glands (x400). Although not directly in the gland, the tissue is itself dysplastic as evident from the distorted histopathology, and localisation of the gene supports the hypothesis that the transcript is somehow associated with the disease state.

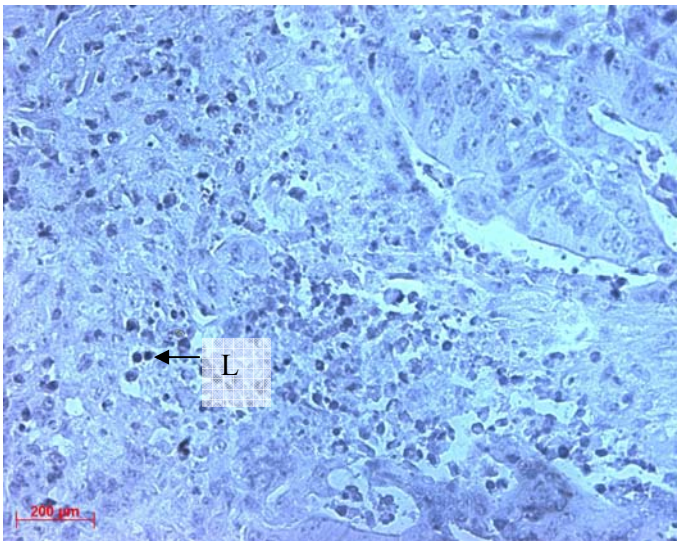


Figure 3.18: Nuclear localisation (L) of the 1.1kb mRNA in a poorly differentiated adenomatous gland and localisation in adjacent dysplastic muscularis mucosa (x400).

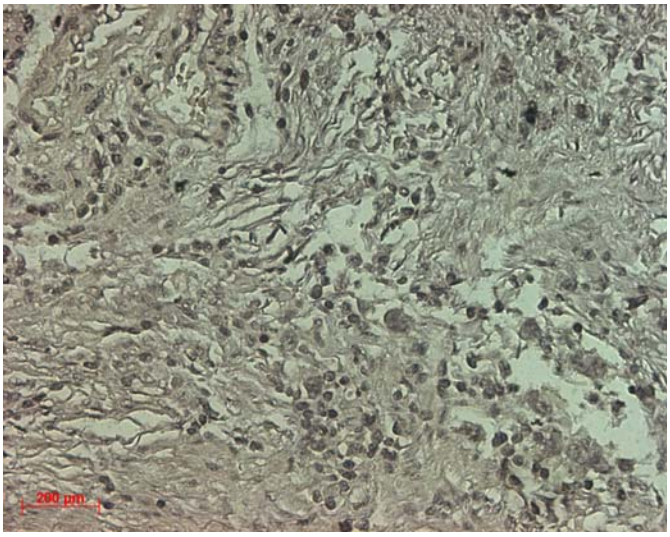


Figure 3.19: Localisation of the 1.1kb mRNA in dysplastic lamina propria (x400).

3.3.1.5 FISH images

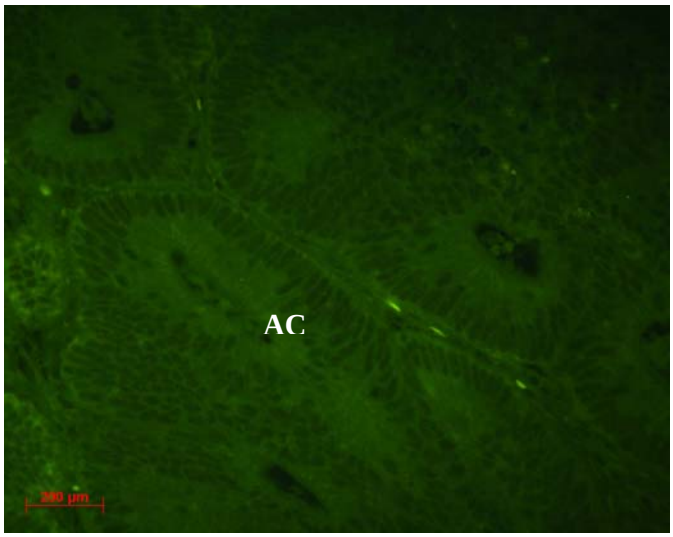


Figure 3.20: Negative control whereby the probe complementary to the 1.1kb DWNN mRNA was omitted from the reaction, hence the lack of fluorescent labelling in the well differentiated adenocarcinoma in the muscularis propria (x400).

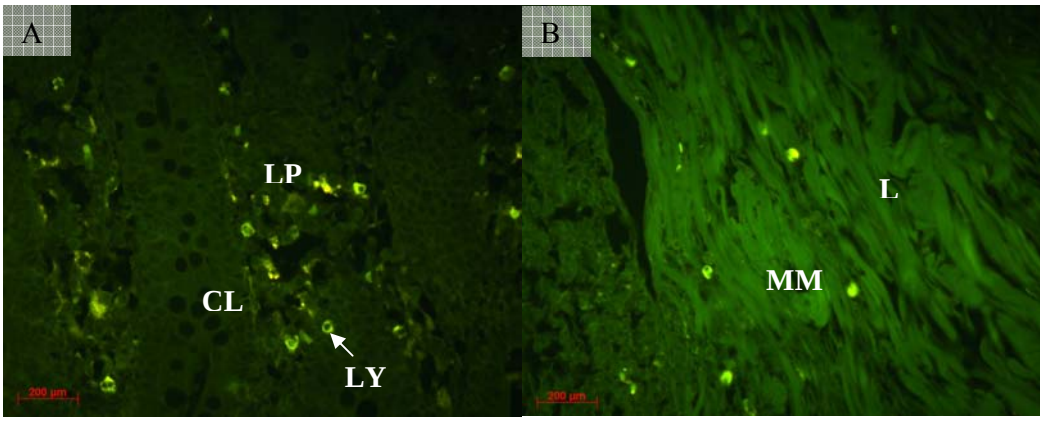


Figure 3.21: Localisation of 1.1kb mRNA in lymphocytes (LY) in normal lamina propria (LP) surrounding crypts of Lieberkühn (CL) (A) and cytoplasmic localisation (L) in adjacent muscularis propria mucosa (MM) (B) (x400).

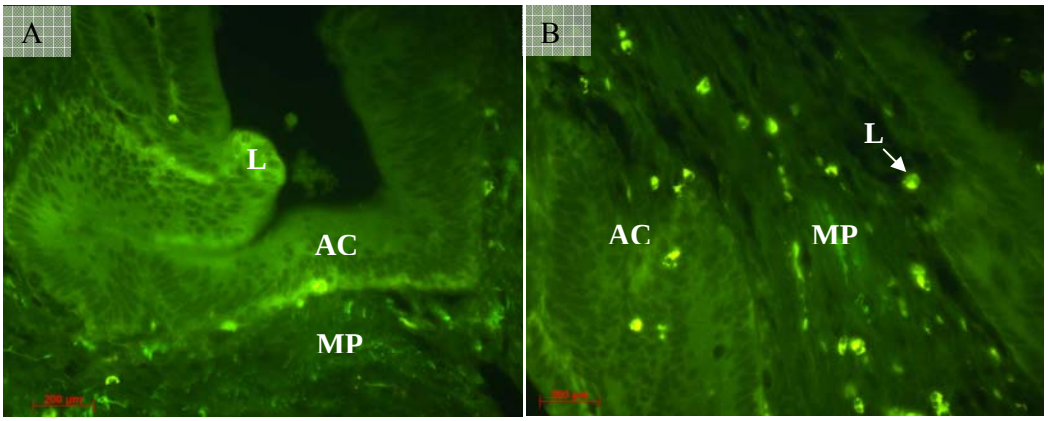


Figure 3.22: In A, cytoplasmic localisation (L) of the 1.1kb mRNA is observed in well differentiated adenocarcinoma (AC) in the muscularis propria (MP), and in B there is cytoplasmic and nuclear localisation in the surrounding muscularis propria (x400).

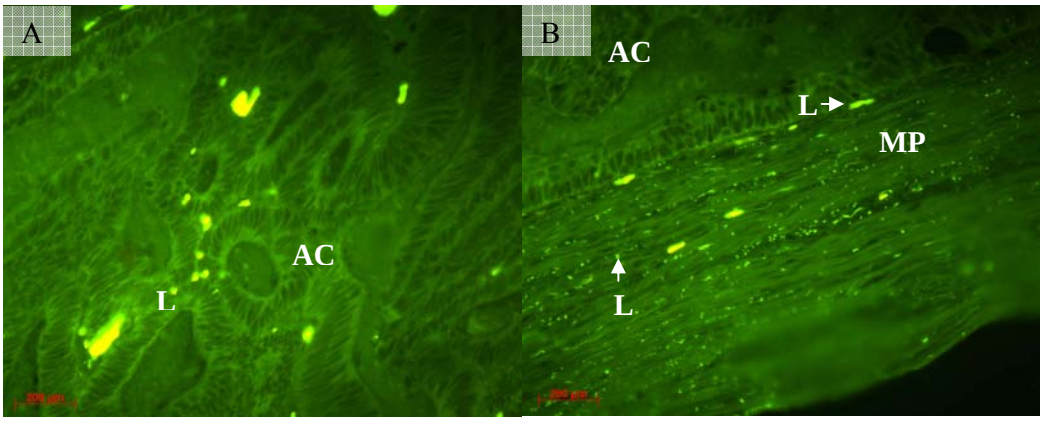


Figure 3.23 A: Cytoplasmic localisation (L) of the 1.1kb mRNA in moderately differentiated adenocarcinoma (AC) in the muscularis propria (MP); B: nuclear and cytoplasmic localisation is also observed in the surrounding muscularis propria (x400).

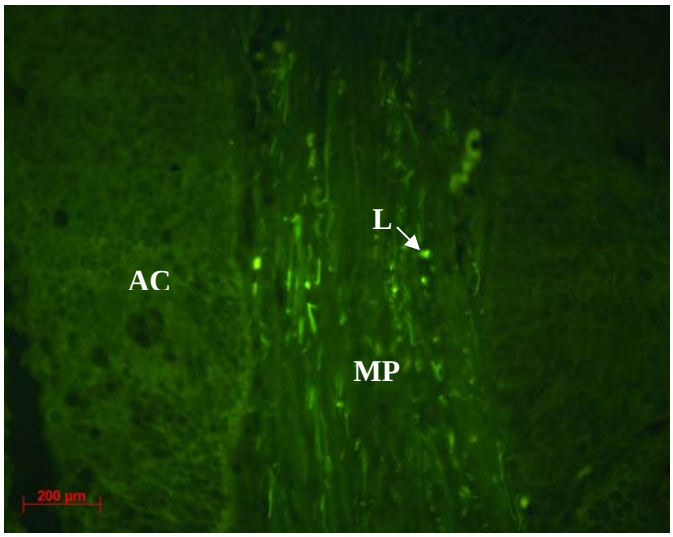


Figure 3.24: Cytoplasmic localisation (L) of the 1.1kb mRNA seen in the muscularis propria (MP). Localisation in the adjacent poorly differentiated adenocarcinoma (AC) was not observed (x400).

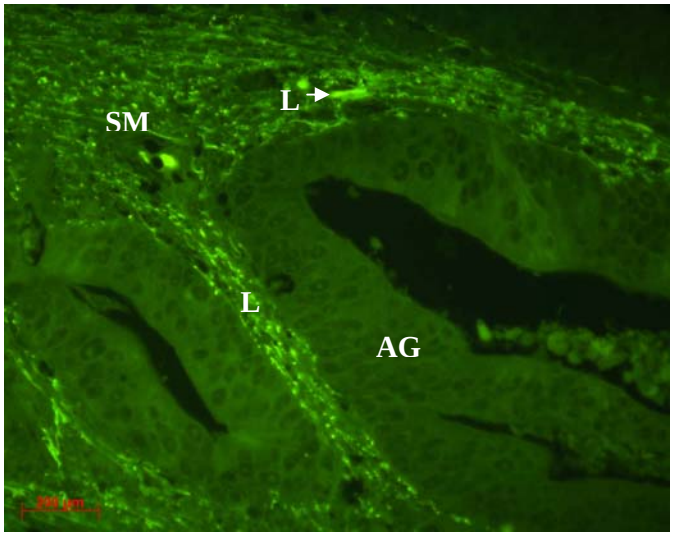


Figure 3.25: Cytoplasmic localisation (L) of the 1.1kb DWNN mRNA in the submucosa (SM) surrounding moderately differentiated adenomatous glands (AG) (x400).

3.3.1.6 Summary

By synthesizing a labelled RNA probe that is only able to bind to the 1.1kb DWNN mRNA, it was concluded by colorimetric and fluorescent means exactly where in the normal and cancerous tissue the mRNA is found. Although the mRNA was localised in normal undiseased colon tissue, the levels were upregulated in cancerous tissue, particularly in the nuclei of lymphocytes in all three grades of the adenomatous glands and the adenocarcinomas. The mRNA was also upregulated in the dysplastic tissues adjacent to these structures and in necrotic debris in the lumen of the glands. As the debris was as a result of cell death, it implies that the gene plays a role in this process. From this experiment it can therefore be concluded that the 1.1kb DWNN mRNA is upregulated in dysplastic structures, tissues and carcinomas in colon cancer.

3.3.2 *In situ* hybridisation of the 6.1kb + E16 mRNA

3.3.2.1 Probe synthesis

The DIG-labelled RNA probe complementary to the 3' end of the 6.1kb mRNA transcript of DWNN i.e. the complete long form of the gene (6.1kb + E16), was synthesized and labelled according to the same procedure as mentioned in 3.3.1. An agarose gel confirmed the insert of interest before labelling was performed. Similar to the probe complementary to the 1.1kb mRNA, this probe was synthesized in the 5' to 3' direction so that it is only able to bind to the 3' end of the complementary DWNN mRNA.

3.3.2.2 Colorimetric ISH images

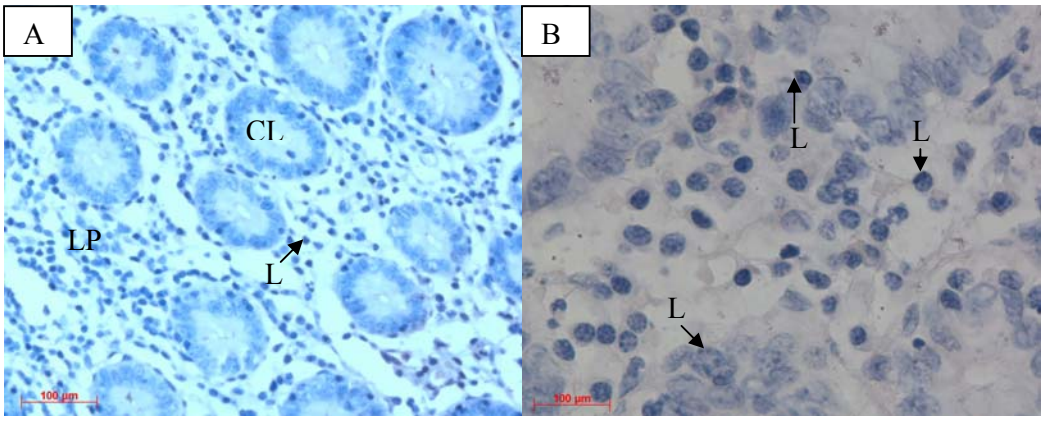


Figure 3.26: Normal colon tissue showing nuclear localisation (L) of 6.1kb + E16 mRNA in lamina propria surrounding crypts of Lieberkühn (CL) (A = x400; B = x1000). Nuclear localisation was also seen under high magnification.

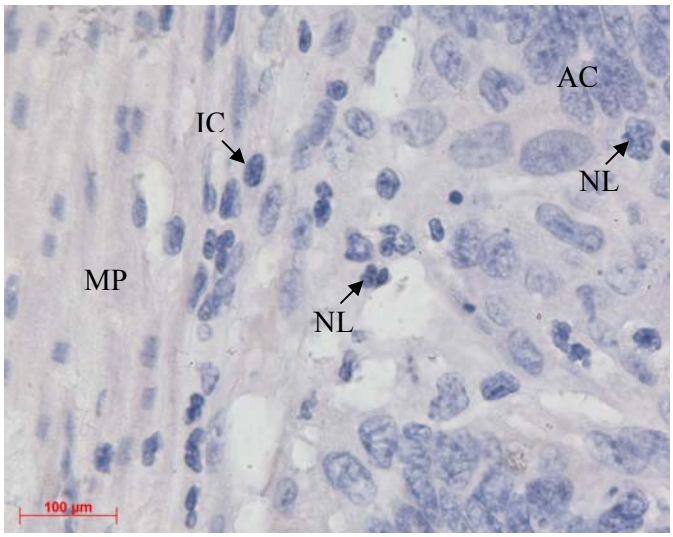


Figure 3.27: Nuclear localisation (NL) of 6.1kb + E16 mRNA in well differentiated adenocarcinoma (AC) in muscularis propria (MP) and in adjacent single invasive cells (IC) (x1000).

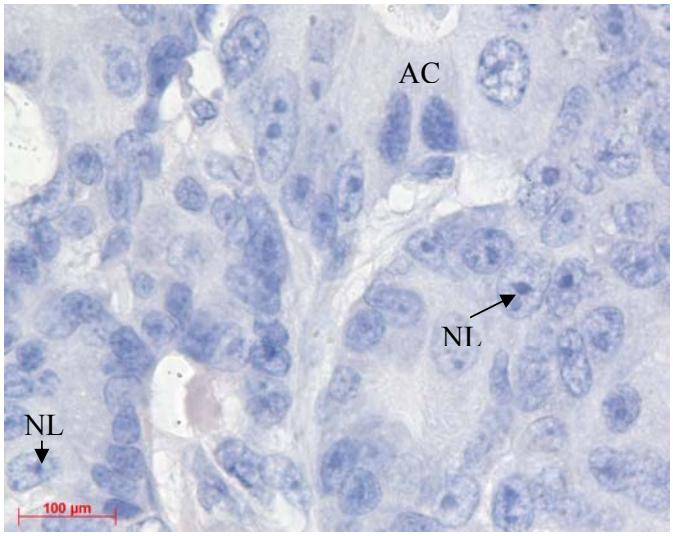


Figure 3.28: Nuclear localisation (NL) of the 6.1kb + E16 mRNA in moderately differentiated adenocarcinoma (AC) in muscularis propria (x1000). Cytoplasmic and nuclear localisation was also seen in adjacent lymphocytes and single invasive cells.

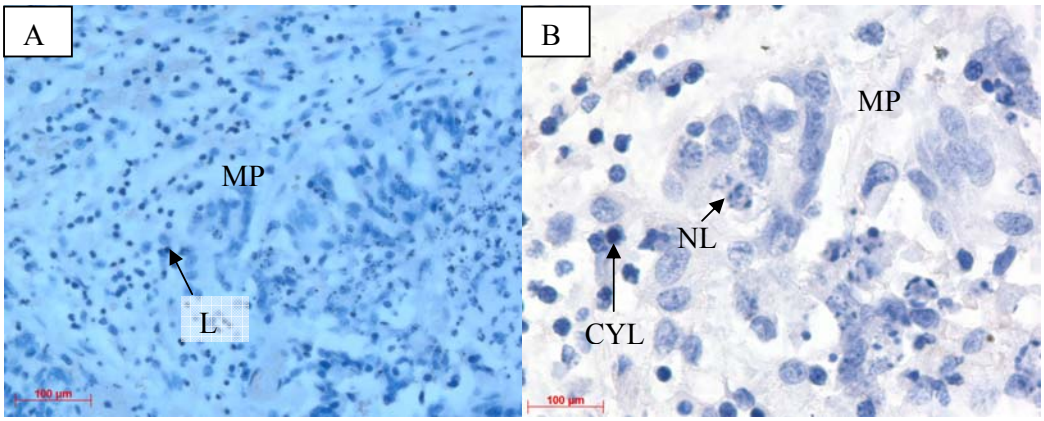


Figure 3.29 A, B: Cytoplasmic (CYL) and nuclear (NL) localisation (L) of the 6.1kb + E16 mRNA transcript in lymphocytes adjacent to poorly differentiated adenocarcinoma in muscularis propria (MP) (A = x400; B = x1000).

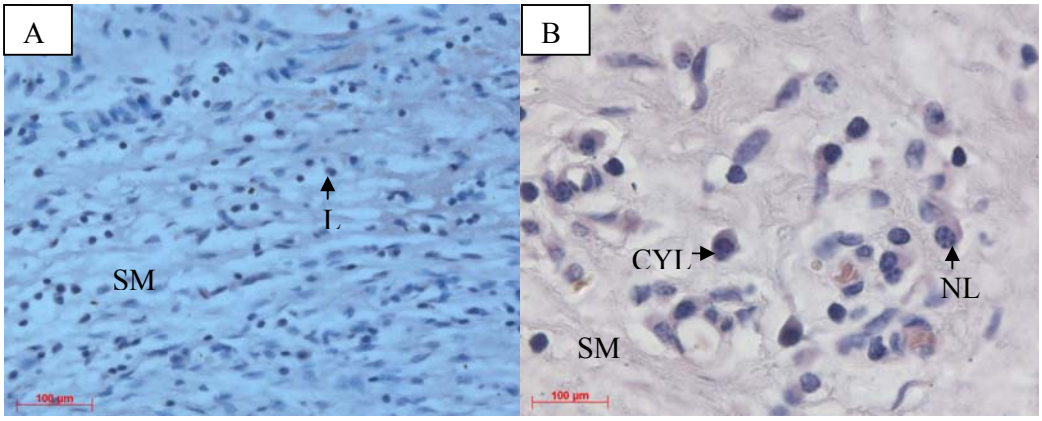


Figure 3.30 A, B: Cytoplasmic (CYL) and nuclear (NL) localisation (L) of 6.1kb + E16 mRNA in lymphocytes (LY) adjacent to moderately differentiated adenomatous glands in submucosa (SM) (A = x400; B = x1000).

3.3.2.3 FISH images

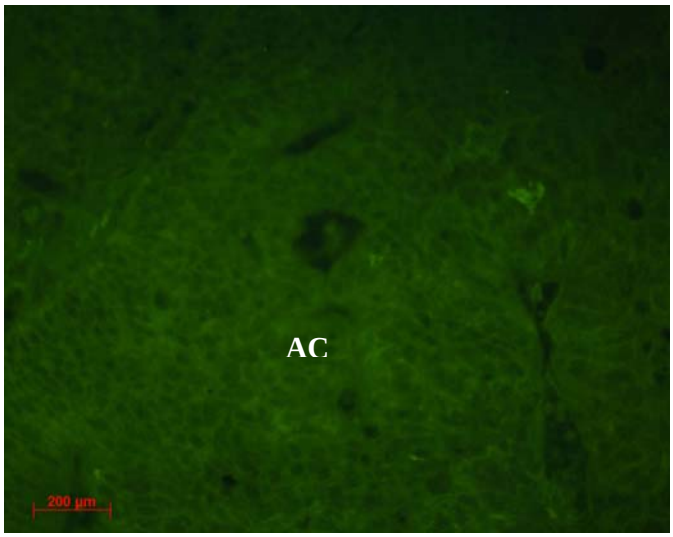


Figure 3.31: Negative control in a poorly differentiated adenocarcinoma case where the probe complementary to the 6.1kb + E16 mRNA was omitted, resulting in no fluorescent labelling (x400).

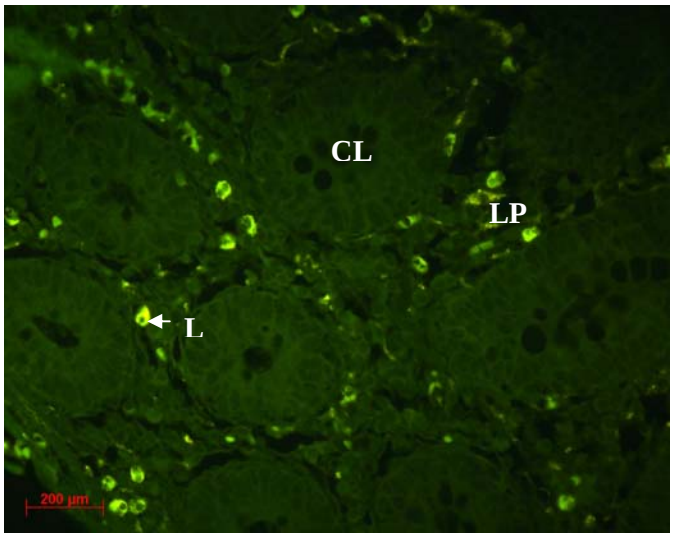


Figure 3.32: Cytoplasmic localisation (L) of 6.1kb + E16 mRNA in lamina propria (LP) in normal tissue in between the crypts of Lieberkühn (CL) (x400).

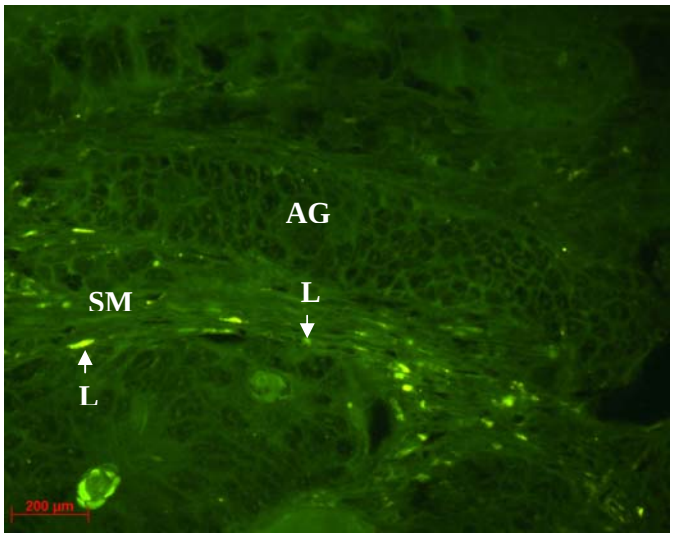


Figure 3.33: Nuclear and cytoplasmic localisation (L) of the 6.1kb + E16 mRNA in the submucosa (SM) surrounding moderately differentiated adenomatous glands (AG) (x400).

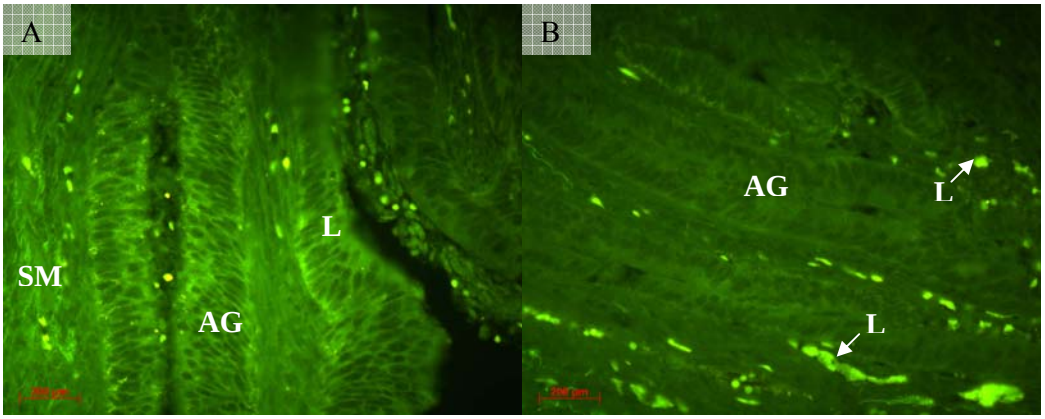


Figure 3.34: In A, cytoplasmic localisation (L) of 6.1kb + E16 mRNA is observed in moderately differentiated adenomatous glands (AG) in the submucosa (SM) and in B, cytoplasmic localisation in surrounding submucosal tissue (x400).

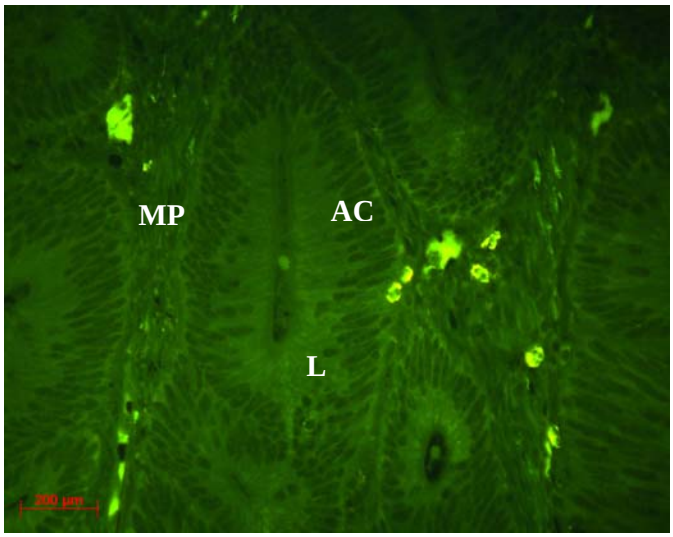


Figure 3.35: Minimal localisation (L) of 6.1kb + E16 mRNA in well differentiated adenocarcinoma (AC) in the muscularis propria (MP) (x400).

3.3.2.4 Summary

The RNA probe complementary to the 3' end of the 6.1kb mRNA localises the complete long form of the DWNN gene. Localisation studies showed an upregulation of this mRNA in colon cancer tissues compared to normal colon tissue, particularly in lymphocytes adjacent to adenomatous glands and adenocarcinomas, as well as in the dysplastic glands and carcinomas. The 1.1kb mRNA, however, showed greater upregulation than the 6.1kb mRNA, and this is an unexpected result as one would anticipate the 6.1kb mRNA to be found at higher levels since this transcript contains the binding sites for the tumour suppressors, p53 and Rb.

3.3.3 *In situ* hybridisation of the exon 16 mRNA

A probe complementary to exon 16 of the 6.1kb + E16 mRNA was synthesized. By comparing the results from ISH of the 6.1kb + E16 mRNA (section 3.2.2) with these results, it is possible to estimate the extent of localisation of the 6.1kb – E16 mRNA.

3.3.3.1 Colorimetric ISH images

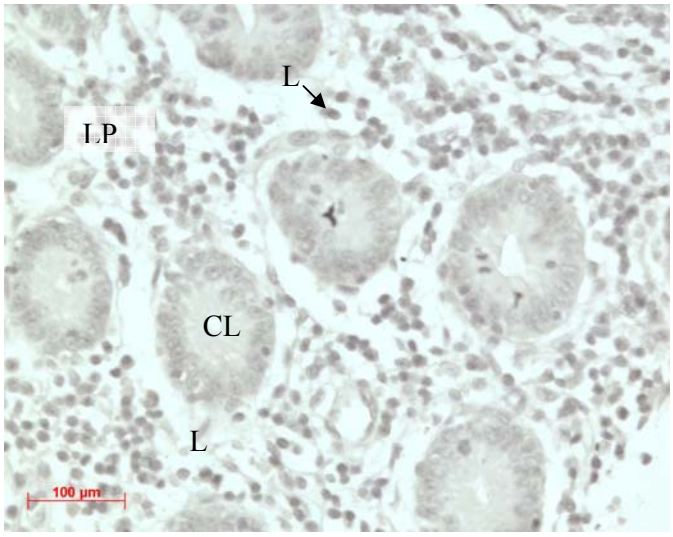


Figure 3.36: Cytoplasmic and nuclear localisation (L) of exon 16 mRNA in lymphocytes in dysplastic lamina propria (LP) surrounding the crypts of Lieberkühn (CL) in normal colon tissue (x400).

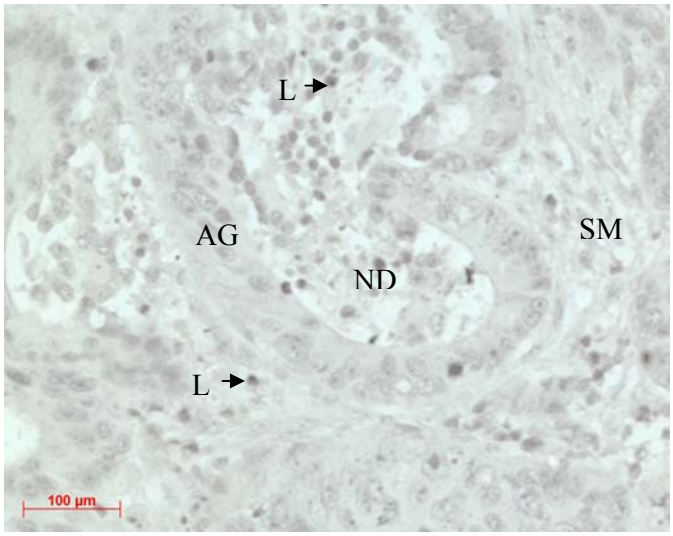


Figure 3.37: Nuclear localisation (L) of the exon 16 mRNA in lymphocytes in the necrotic debris (ND) in the lumen of a well differentiated adenomatous gland (AG) and in the submucosa (SM) (x400).

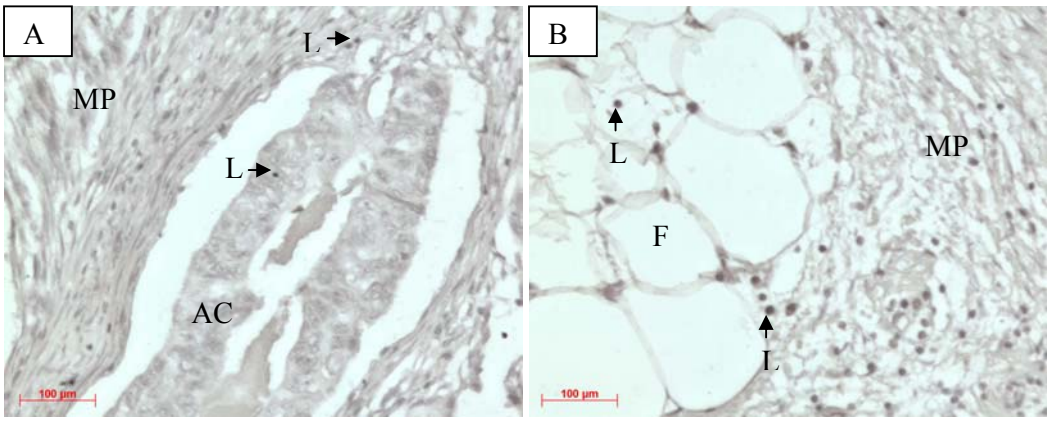


Figure 3.38A: Cytoplasmic and nuclear localisation (L) of exon 16 mRNA in moderately differentiated adenocarcinoma (AC) and in the surrounding muscularis propria (MP); B: cytoplasmic and nuclear localisation (L) in lymphocytes in the fat cells (F) of the subserosa and in the muscularis propria (MP) (x400).

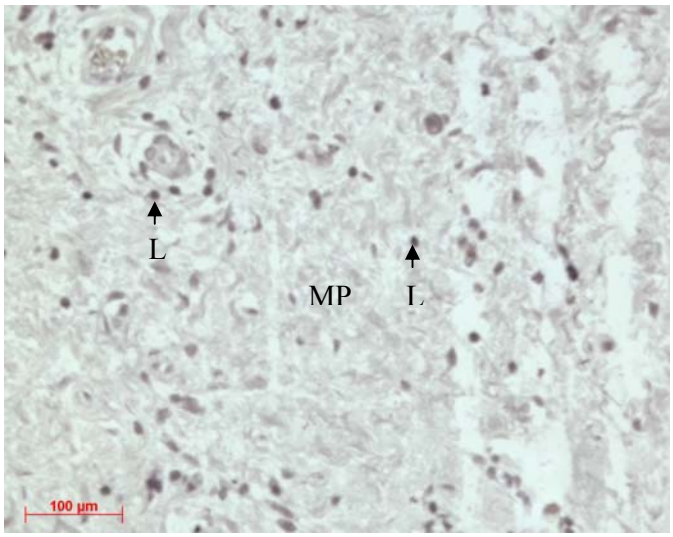


Figure 3.39: Localisation (L) in lymphocytes in dysplastic muscularis propria (MP) adjacent to well differentiated adenocarcinoma (x400).

3.3.3.2 Summary

The exon 16 mRNA showed localisation mainly in the lymphocytes occurring in well and moderately differentiated adenocarcinomas, well differentiated adenomatous glands and their surrounding dysplastic tissue. Localisation was also observed in necrotic debris found within the lumens of adenomatous glands. Although it is difficult based on this "subtractive" method to estimate the localisation of the 6.1kb – E16 mRNA this way, this experiment allowed for the identification of sites of localisation of the exon 16 mRNA and when correlated with the results from quantitative PCR, it serves to confirm to a degree that the 6.1kb – E16 mRNA is found at lower levels in cancerous tissue than the 6.1kb + E16 mRNA. This suggests that exon 16 plays a significant role in the functioning of the 6.1kb mRNA in diseased tissues.

3.4 Immunocytochemistry (DWNN)

This technique was used to localize the expression of the DWNN gene, to establish in which parts of the tissue the proteins were expressed, and to what extent, if any, they were upregulated in diseased tissues compared to normal tissue. Immunocytochemistry determines the localization of DWNN proteins by using the labelled rabbit antihuman DWNN primary antibody to mark specific antigens found on the protein. The biotinylated mouse antirabbit secondary antibody, which reacts with peroxidase conjugated streptavidin, binds to the primary antibody, which has attached itself to the complementary DWNN protein. The chromogen 3, 3'-diaminobenzidine (DAB) is cleaved by peroxidase, resulting in a brown-coloured precipitate developing at the site of the antigen location.

Biotin is a small molecule that is strongly bound by the avidin and streptavidin proteins. The preformed complex used here consists of a molecule of streptavidin with three molecules of biotin-labelled peroxidase. As the secondary antibody is also labelled with biotin, the free biotin-binding site on the streptavidin binds to this antibody. This results in three molecules of the streptavidin-peroxidase complex bound per biotin-labelled antibody. Endogenous biotin that may be present is blocked by heat treatment for more accurate results.

Two different antibodies are used: 1 is specific for the protein expressed by the 5' end of the 1.1kb mRNA i.e. the 5' end of the 13kDa protein and will therefore localise to all DWNN proteins; the second is specific only to the protein expressed by the RBBP6 region of the 6.1kb mRNA and will only localise these proteins.

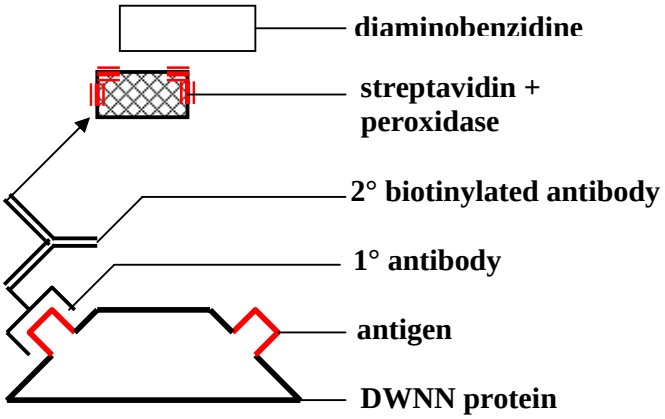


Figure 3.40: Diagrammatic representation of the reactions involved in immunocytochemistry

3.4.1 Localisation of the DWNN protein

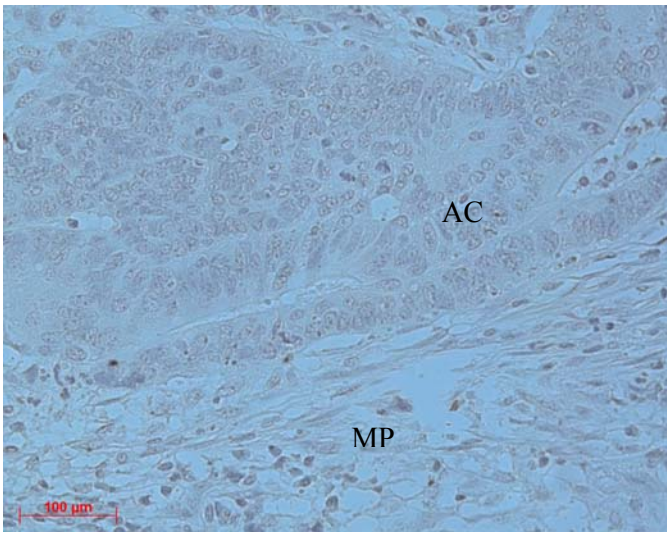


Figure 3.41: Negative control where diaminobenzidine has been excluded, hence the lack of labelling (x400). This section shows well differentiated adenocarcinoma (AC) in the muscularis propria (MP).

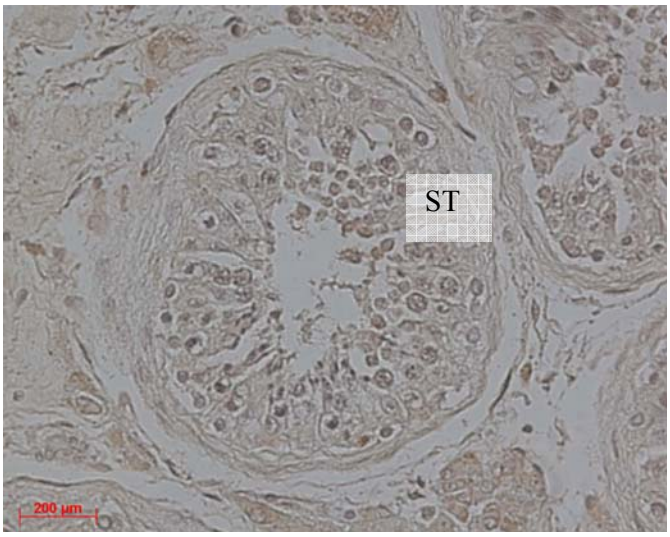


Figure 3.42: Positive control showing nuclear and cytoplasmic localisation of the DWNN protein in the semeniferous tubules (ST) of the testes (x400). As it has previously been ascertained that DWNN is highly expressed in this tissue, this is the standard to which experimental results are compared to determine if labelling is positive.

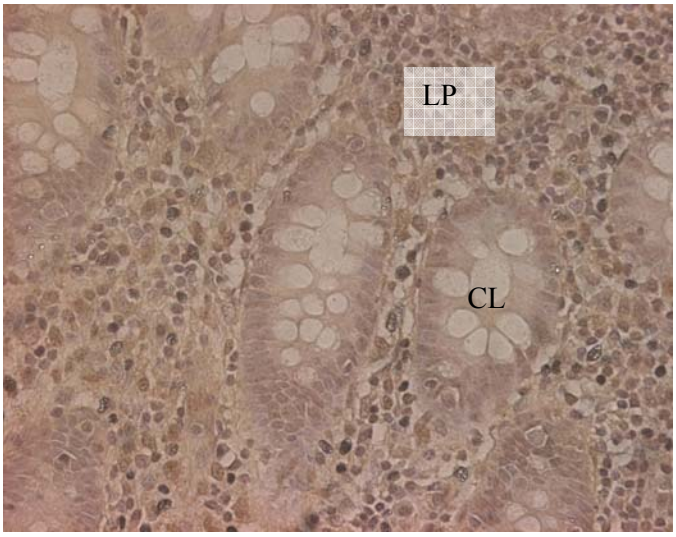


Figure 3.43: Normal undiseased colon showing nuclear and cytoplasmic localisation of the DWNN protein in lamina propria (LP) in between the crypts of Lieberkühn (CL) (x400) indicating that the gene is expressed in normal tissue.

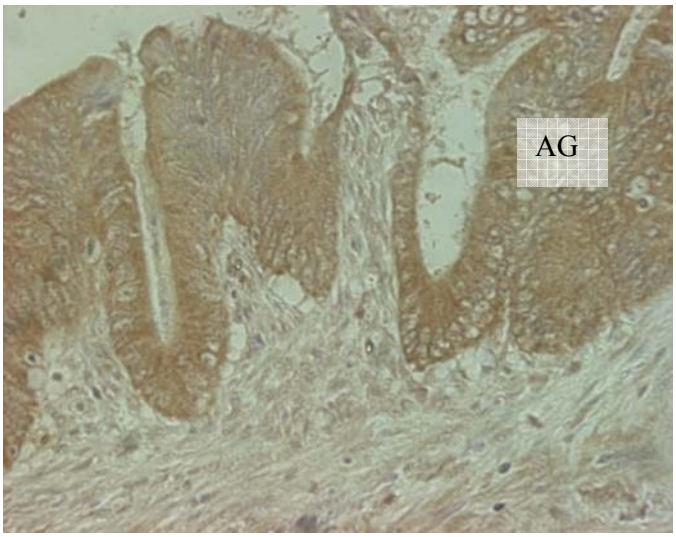


Figure 3.44: Cytoplasmic localisation of the DWNN protein in poorly differentiated adenomatous glands (AG) in the lamina propria (x400). Expression of the gene in dysplastic tissues further verifies its involvement in cancer progression.

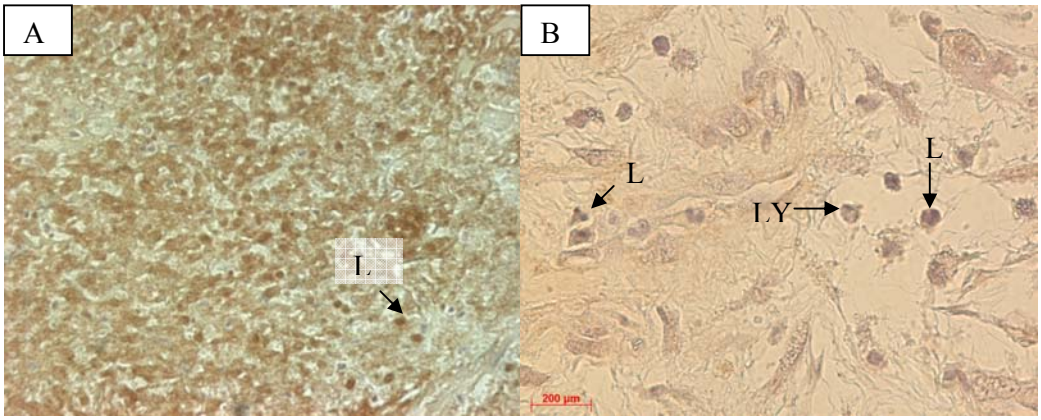


Figure 3.45A, B: Cytoplasmic localisation (L) of the DWNN protein in lymphocytes (LY) (A = x400; B = x1000).

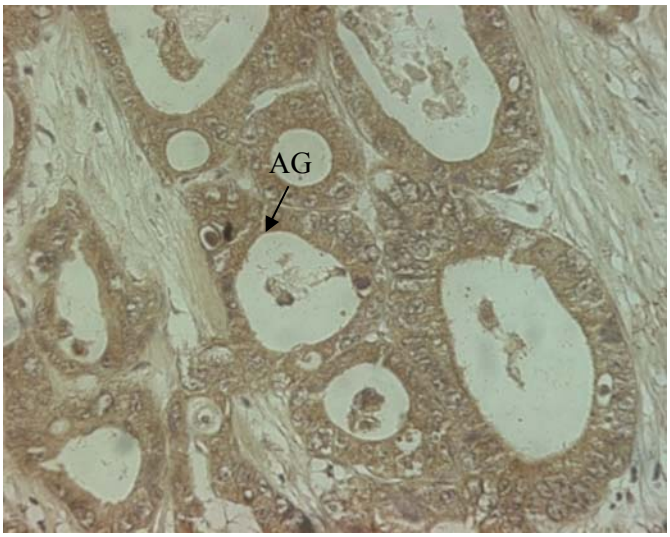


Figure 3.46: Nuclear and cytoplasmic localisation of the DWNN protein in well differentiated adenomatous glands (AG) in submucosa (x400), suggesting that DWNN expression has a significant relationship with dysplastic tissue. As in ISH, it can only be determined if the protein aids in cancer progression or inhibits it after examining the results from apoptosis detection.

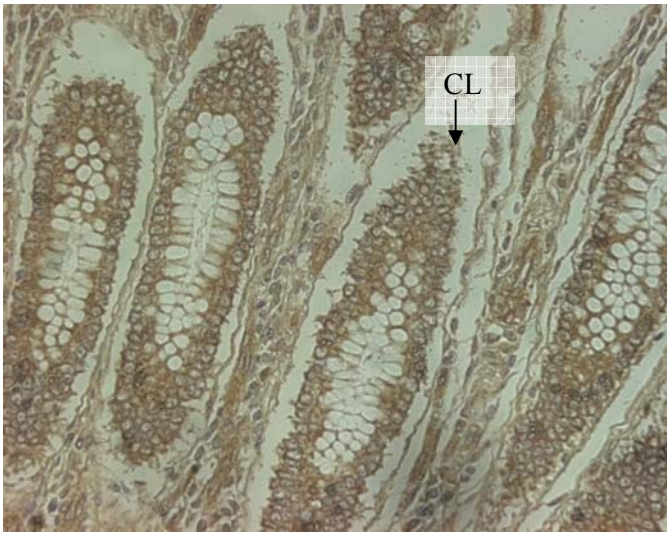


Figure 3.47: Cytoplasmic localisation of the DWNN protein in the crypts of Lieberkühn (CL) in dysplastic lamina propria (x400). The protein is expressed in the proliferative zone of the crypts (i.e. the lower third).

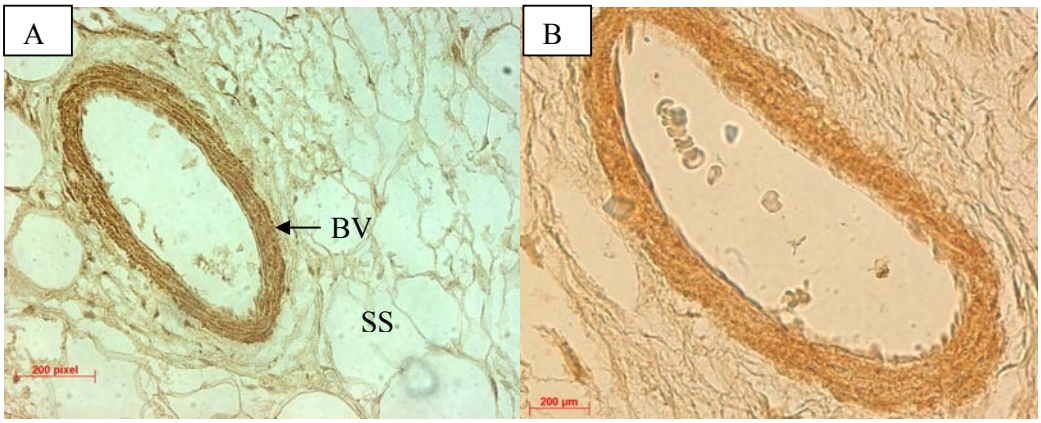


Figure 3.48A, B: Cytoplasmic localisation of the DWNN protein in blood vessel (BV) in subserosa (SS) (A = x400; B = x1000).

3.4.2 Localisation of the RBBP6 protein

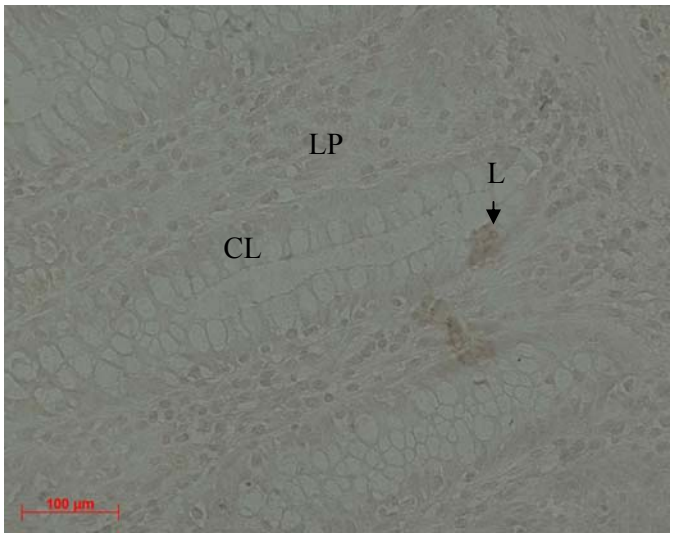


Figure 3.49: Cytoplasmic localisation (L) of the RBBP6 protein in the crypts of Lieberkühn (CL) in the lamina propria (LP) of normal colon tissue (x400).

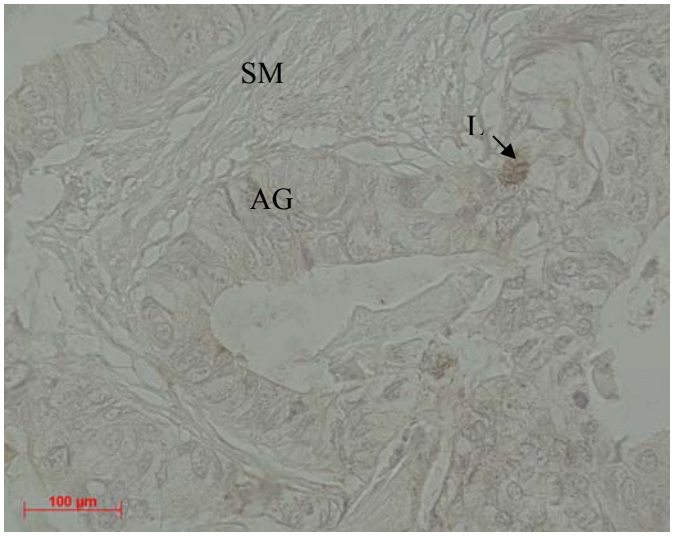


Figure 3.50: Cytoplasmic localisation (L) of the RBBP6 protein in moderately differentiated adenomatous glands (AG) in the submucosa (SM) (x400).

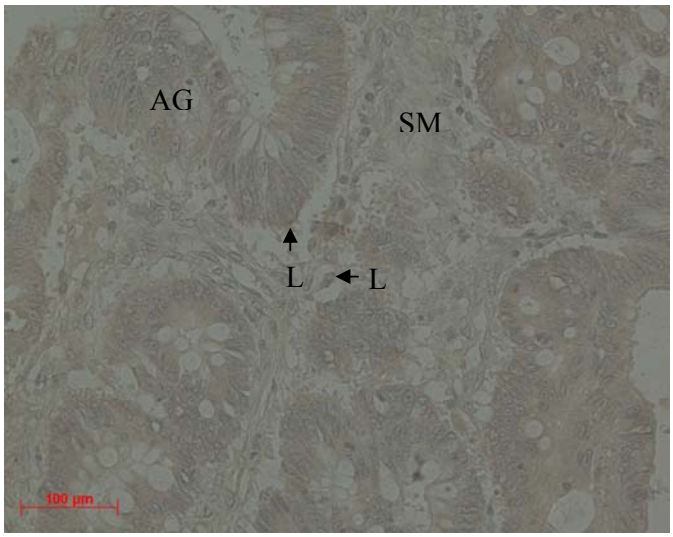


Figure 3.51: Cytoplasmic localisation (L) of the RBBP6 protein in well differentiated adenomatous glands (AG) and in the surrounding submucosa (SM) (x400).

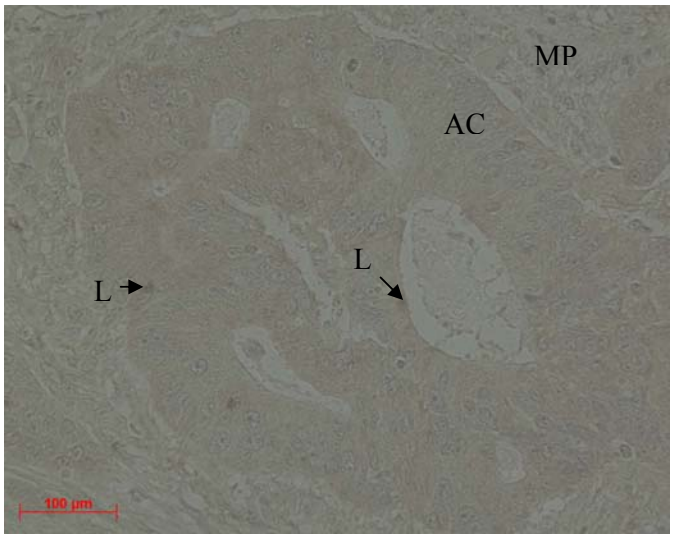


Figure 3.52: Cytoplasmic localisation (L) of the RBBP6 protein in moderately differentiated adenocarcinoma (AC) in the muscularis propria (MP) (x400).

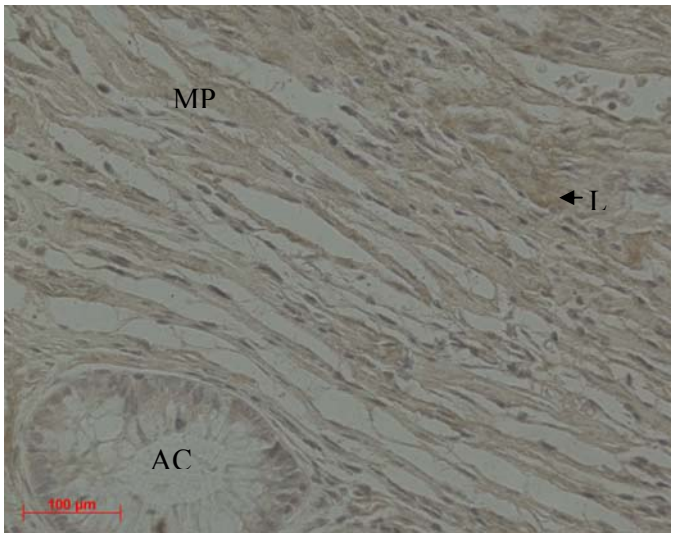


Figure 3.53: Cytoplasmic localisation (L) of the RBBP6 protein in the muscularis propria (MP) surrounding well differentiated adenocarcinoma (AC) (x400).

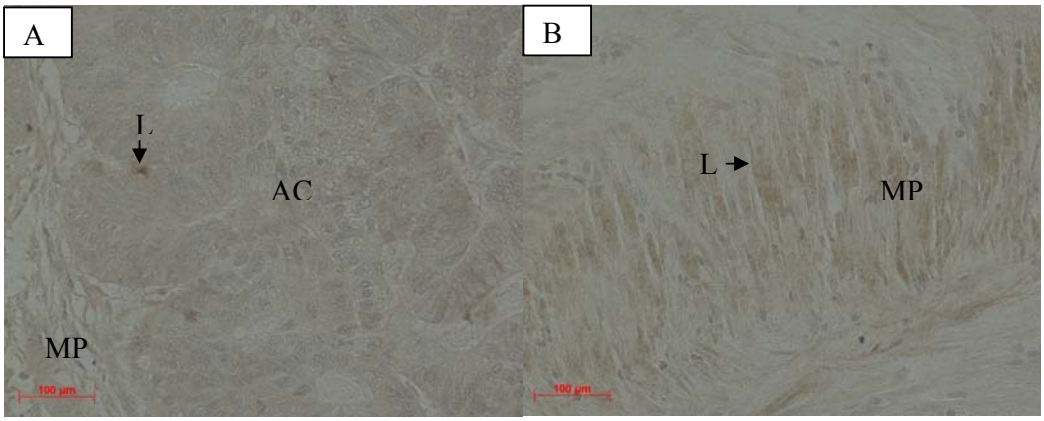


Figure 3.54: Cytoplasmic localisation (L) of the RBBP6 protein in well differentiated adenocarcinoma (AC) in A; and in B cytoplasmic localisation (L) of the RBBP6 protein in the surrounding muscularis propria (MP) (x400).

3.4.3 Image analysis of DWNN protein

Image analysis was performed to determine the labelling intensity of diaminobenzidine localising the DWNN protein in nine colonic structures. A digital image is made up of pixels with a grey scale ranging from 1 to 256. Diaminobenzidine labelling falls between 160 and 256. Areas of structures showing labelling were divided into three or four sectors to attain a higher *n* value and the total number of pixels representing high immunolabelling was calculated per μm^2. The average for each structure was then calculated and this was then multiplied by 100 to obtain a comprehensible value. The average deviations were also calculated, denoted by error bars in the graph (**figure 3.55**)

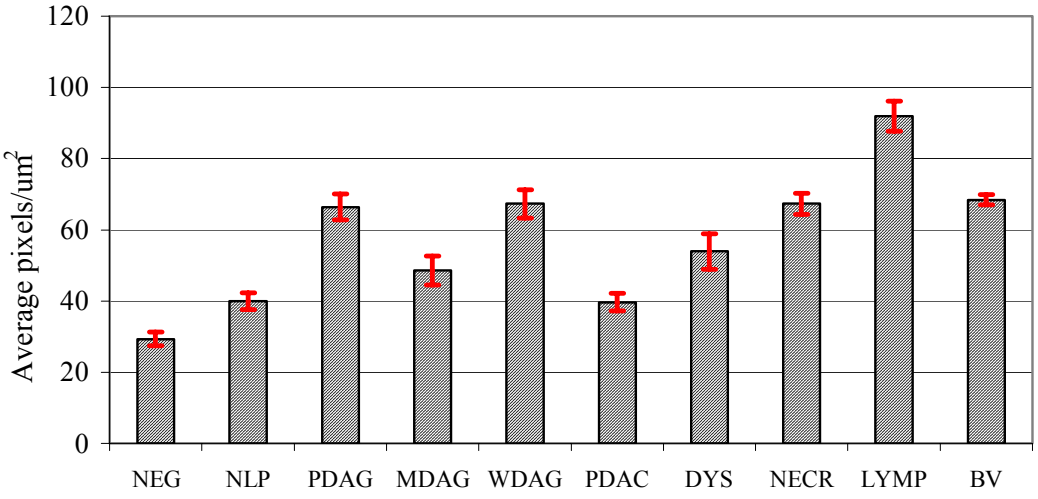


Figure 3.55: Average immunolabeling of DAB per area

NEG – negative control

NLP – normal epithelial tissue

PDAG – poorly differentiated adenomatous glands

MDAG – moderately differentiated adenomatous glands

WDAG – well differentiated adenomatous glands

PDAC – poorly differentiated adenocarcinoma

DYS – dysplastic tissue

NECR – necrotic debris

LYMP – lymphocytes

BV – blood vessel

From this analysis it can be ascertained in which structures the DWNN protein is upregulated in comparison to the negative control and the normal colon tissue. Diaminobenzidine labelling was seen in the negative control, suggesting that the secondary antibody in conjunction with peroxidase, has resulted in the cleavage of this substrate. However, results can still be interpreted by subtracting this value of the negative control from the labelling of each structure. To determine the level of protein upregulation, labelling in each structure is compared to that of the normal colon tissue, as denoted by NET (normal epithelial tissue). From this, it can be observed that there was no upregulation of the DWNN protein in poorly differentiated adenocarcinomas (PDAC) as it showed the same level of labelling as the normal tissue.

There was a slight upregulation in the moderately differentiated adenomatous glands (MDAG) and even more upregulation in the dysplastic tissues (DYS). Significantly more upregulation of the protein could be seen in the poorly (PDAG) and well differentiated adenomatous glands (WDAG), necrotic debris (NECR) and the blood vessels (BV). The highest level of upregulation of the DWNN protein was shown to be in the lymphocytes (LYMP), as also observed in the images.

3.4.4 Summary

Immunocytochemistry allowed for the DWNN protein to be localised in normal and cancerous tissues and from this it was observed that there was an upregulation of DWNN expression in cancerous structures and their neighbouring dysplastic tissues. Not only was the expression particularly significant in dysplastic structures, such as adenocarcinoma, but it was only seen in the proliferative zone of the crypts in dysplastic lamina propria implying that these proliferating cells were expressing the gene in the abnormal tissue. Image analysis showed increased upregulation in well and poorly differentiated adenomatous glands, necrotic debris, blood vessels and in the lymphocytes.

Localisation of the protein expressed by the RBBP6 region of the 6.1kb DWNN mRNA did not show a level of upregulation as high as that of the DWNN protein. Low levels of localisation were observed in moderately and well differentiated adenomatous glands and moderately and well differentiated adenocarcinomas, as well as in the surrounding dysplastic tissues. These results suggest that although the RBBP6 protein is found in these cancerous structures, it is the 13kD DWNN protein that plays the major role in colon cancer.

With ISH and ICC it can only be concluded that the mRNA and the DWNN protein are upregulated in dysplastic structures and tissues, but at this point it cannot be said what role DWNN plays in cancer progression.

3.5 TUNEL

Terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate biotin nick-end labelling, or TUNEL, was applied to visualise where in the tissues apoptosis was occurring. By comparing the results from *in situ* hybridisation and immunocytochemistry, it could then be ascertained if DWNN expression was directly correlated with the apoptotic process. If a significant relationship was found, it could be deduced whether the gene (and the particular transcript probed for) was pro- or anti-apoptotic.

During apoptosis endogenous endonucleases result in the formation of DNA fragments which are partially responsible for the morphological changes seen in the nuclei of apoptotic cells. These fragments are in the region of 180 to 200 base pairs. The tissue sections are first treated with Proteinase K, which digests cross-linked proteins to increase cell permeability and contact with the DNA. The DeadEnd™ Colorimetric TUNEL system adds biotinylated deoxyuridine at the 3'-OH DNA ends of each fragment by application of the enzyme, terminal deoxynucleotidyl transferase (TdT). The nontemplate-dependent DNA polymerase, Tdt, acts by catalysing the incorporation of deoxyribonucleotide triphosphates to the 3' ends of double or single-stranded DNA. Horseradish-peroxidase-labelled streptavidin binds to the biotinylated nucleotides. Addition of the peroxidase substrate, hydrogen peroxide, and the diaminobenzidine (DAB) chromogen result in apoptotic nuclei staining dark brown.

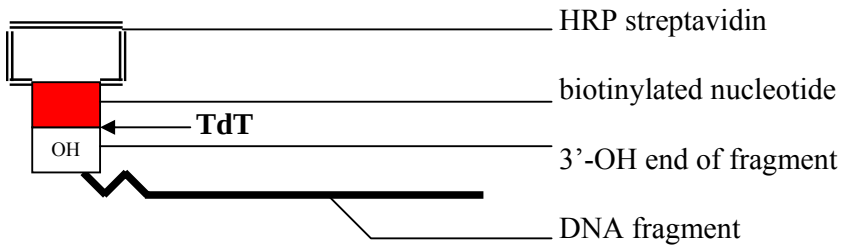


Figure 3.56: Diagrammatic representation of reactions occurring in TUNEL

3.5.1 TUNEL images

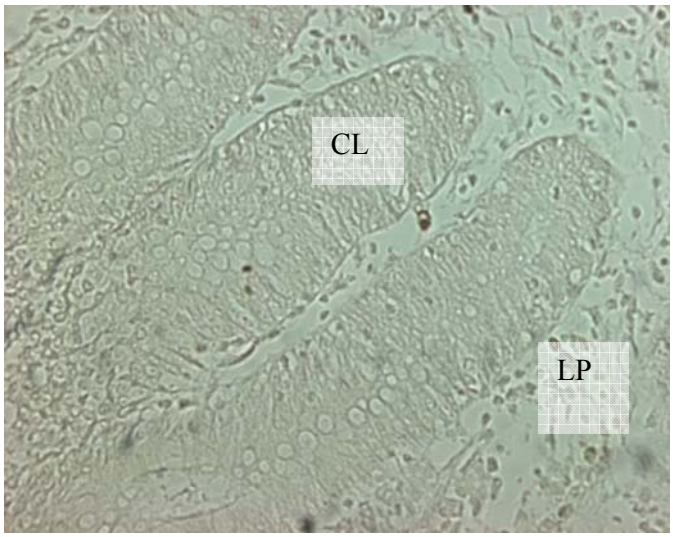


Figure 3.57: Normal undiseased tissue showing few labelling sites in crypts of Lieberkühn (CL) in the lamina propria (LP) (x400).

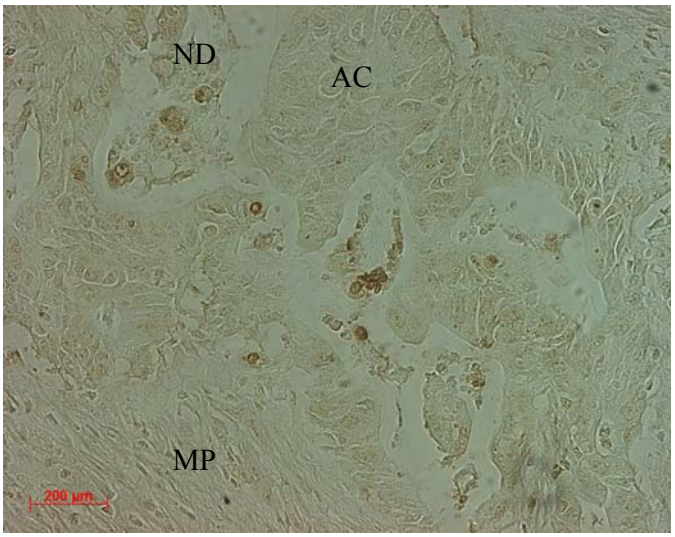


Figure 3.58: Nuclear labelling in poorly differentiated adenocarcinoma (AC) in the muscularis propria (MP) and nuclear and cytoplasmic labelling in necrotic debris (ND) (x400), indicating sites of apoptosis. As nuclear and cytoplasmic localisation of the DWNN transcript was observed here as well, it can be deduced that this transcript is a pro-apoptotic gene.

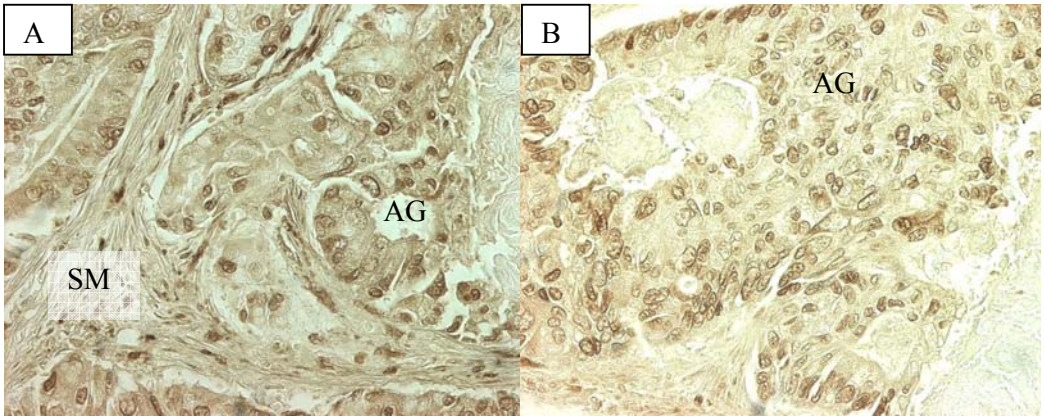


Figure 3.59A, B: Nuclear and cytoplasmic labelling in moderately differentiated adenomatous glands (AG) in the submucosa (SM) (x400). In ISH nuclear and cytoplasmic labelling was also observed here, further evidence of the transcript being pro-apoptotic. Localisation of the DWNN protein in this case was seen in the nuclei of an adenocarcinoma in the subserosa.

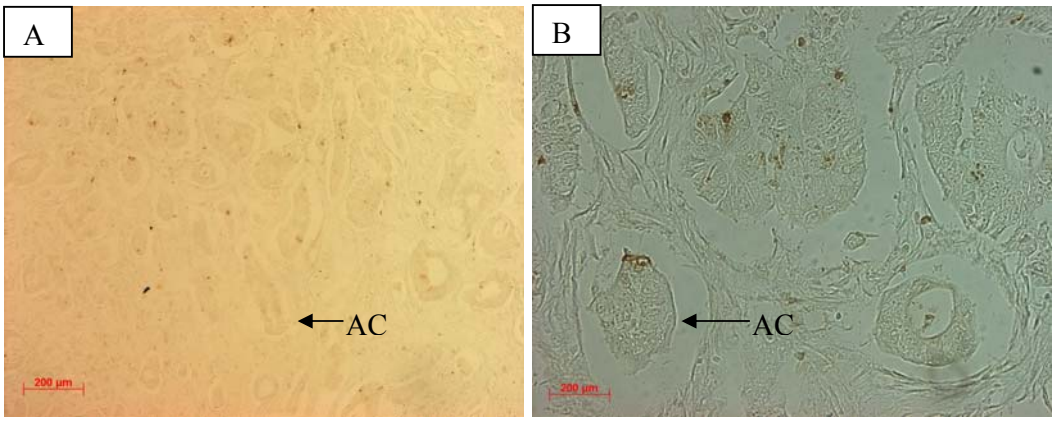


Figure 3.60: Nuclear labelling in moderately differentiated adenocarcinoma (AC) in muscularis propria (A= x100; B= x400). Immunocytochemistry showed DWNN protein expression in the same area, thereby indicating that it is pro-apoptotic.

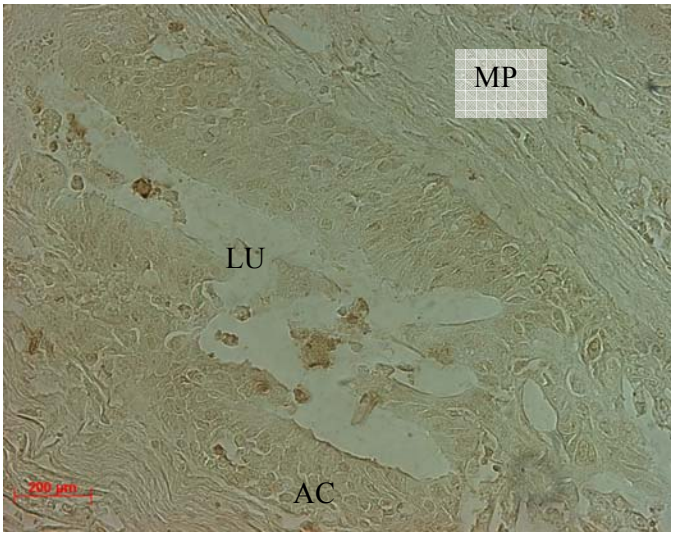


Figure 3.61: Nuclear and cytoplasmic labelling in necrotic debris in the lumen (LU) of a moderately differentiated adenocarcinoma (AC) in the muscularis propria (MP) (x400). mRNA localisation showed the transcript in the adenocarcinoma nuclei and in adjacent lymphocytes. The protein expressed in the cytoplasm of these dysplastic glands.

3.5.2 Summary

The TUNEL method labels DNA fragments and is therefore an effective technique for apoptosis detection. By comparing the results obtained from ISH and ICC, it could be concluded that apoptosis was occurring in the same regions as the 13kDa mRNA and DWNN protein, namely in the adenocarcinomas, adenomatous glands and necrotic debris in the lumens of dysplastic glands. Localisation was also seen in lymphocytes adjacent to adenocarcinomas, as these T-cells are involved in cell death. TUNEL therefore allows for conclusions to be drawn as to the role of DWNN in cancerous tissue, which is pro-apoptotic.

3.6 Ki-67 proliferation assay

This technique was applied to ascertain the site of proliferation in the cancerous colon tissue. Proliferation normally occurs in the lower third of the crypts of Lieberkühn. One would expect to see extensive proliferation in dysplastic structures such as the adenomatous glands. Proliferation markers are helpful in allowing for differentiation between rapidly growing tumours from slower growing tumours (Saeger, 2004). Cells in the G1, G2, S and M-phase of the cell cycle express the Ki-67 antigen and this is a commonly used proliferative marker which has been shown to have a substantial association with invasiveness (Ishida *et al.* 2004; Zhao *et al.* 1999). The Ki-67 goat polyclonal immunoglobulin-G (Santa Cruz Biotechnology) was used in conjunction with a biotinylated donkey anti-goat immunoglobulin-G antibody and a biotinylated staining system to produce a colorimetric reaction.

3.6.1 Ki-67 images

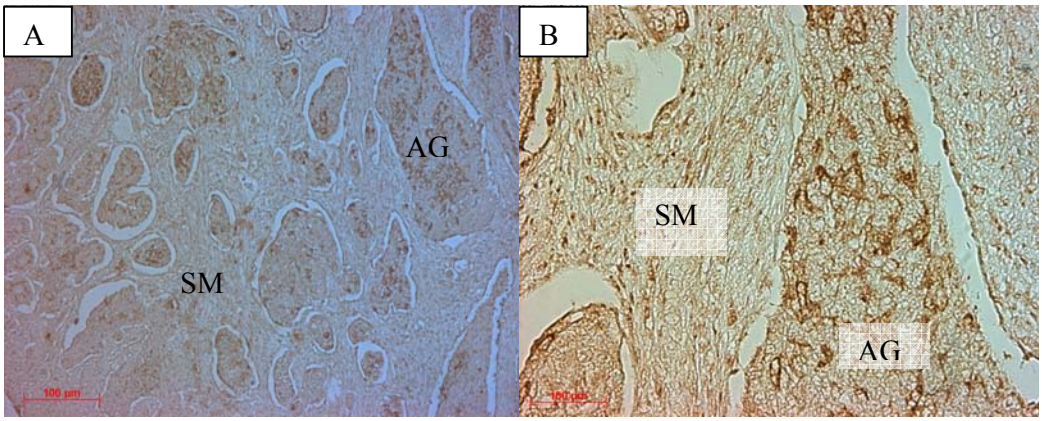


Figure 3.62A, B: Cytoplasmic and nucleic proliferation occurring in poorly differentiated adenomatous glands (AG) in the submucosa (SM) indicating cell division in the carcinomatous tissue (A = x100; B = x400).

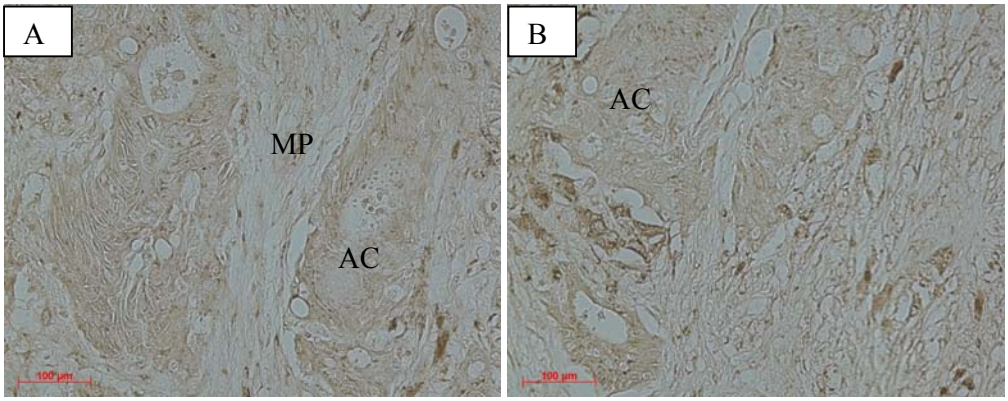


Figure 3.63A & B: Nuclear staining indicating nuclear proliferation in poorly differentiated adenocarcinoma (AC) in the muscularis propria (MP) (x400). Nuclear proliferation is also seen in the dysplastic muscularis propria.

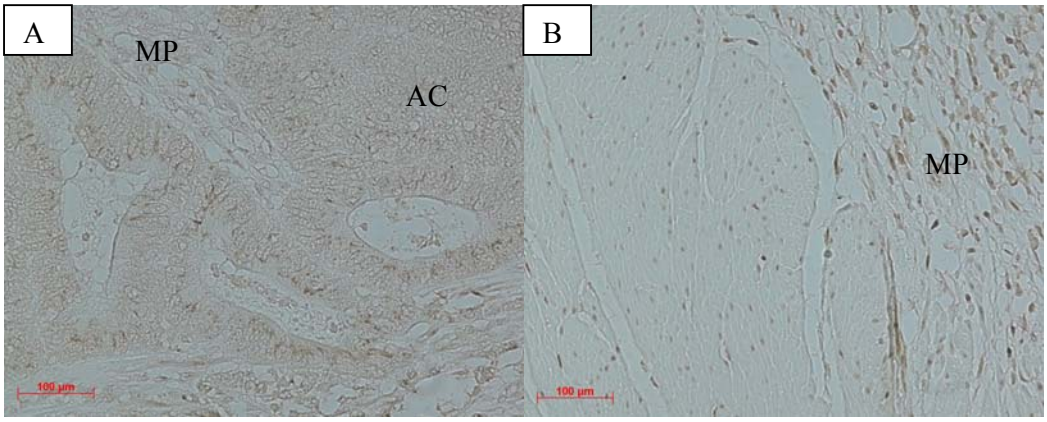


Figure 3.64: Nuclear localisation in moderately differentiated adenocarcinoma (AC) in muscularis propria (MP) (A) and nuclear localisation in dysplastic muscularis propria (B) (x400).

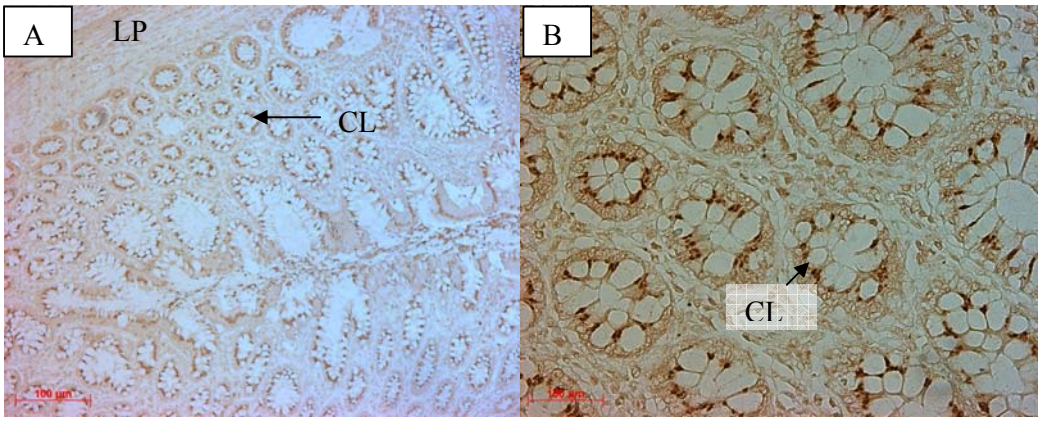


Figure 3.65A, B: Ki-67 localisation in nuclei of crypts of Lieberkühn (CL) and nuclear staining in interstitial cells of the lamina propria (LP) in well differentiated adenocarcinoma (A = x100; B = x400).

3.6.2 Summary

From this assay, it could be seen that proliferation was heightened in poorly, moderately and well differentiated adenomatous glands. This indicates increased cell division of cancerous cells, typical of this disease. However, mRNA and protein localisation was not correlated with proliferation in the same area and as apoptosis is occurring simultaneously with gene expression, this suggests that the gene is pro-apoptotic.

3.7 Bcl-2 assay

Bcl-2 is an inhibitor of apoptosis and overexpression of this oncoprotein occurs commonly in colon cancer (Yang *et al.* 1999). The aim of this experiment was to verify if DWNN was pro-apoptotic, as deduced from TUNEL, or if Bcl-2 was localised at the same sites as the mRNA and protein. The Bcl-2 mouse monoclonal immunoglobulin-G (Santa Cruz Biotechnology) was used in conjunction with a biotinylated goat anti-mouse immunoglobulin-G antibody and a biotinylated staining system, which resulted in a colorimetric reaction.

3.7.1 Bcl-2 images

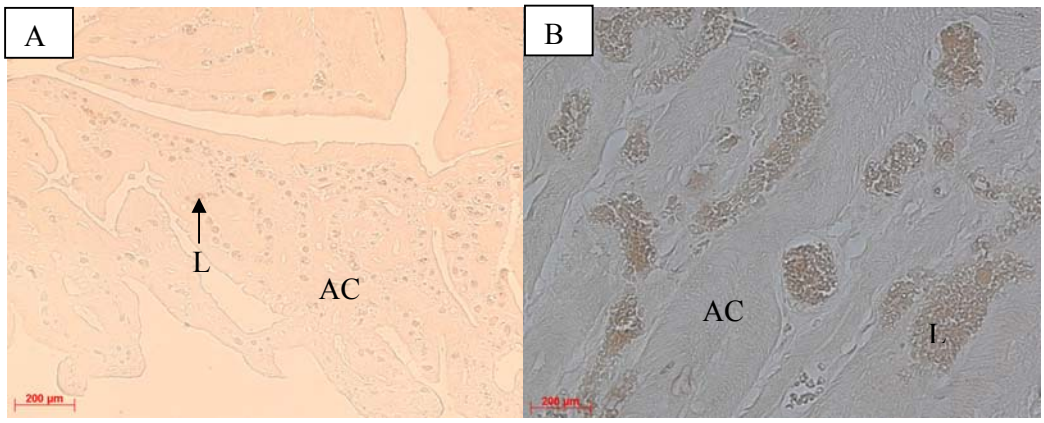


Figure 3.66A, B: Bcl-2 localisation (L) in tissue in between well differentiated adenocarcinoma (AC) in the muscularis propria (A = x100; B = x400). As Bcl-2 is an inhibitor of apoptosis and the gene was not found to be upregulated in this area, both by ISH and by ICC, it can be concluded that this particular transcript of DWNN is pro-apoptotic.

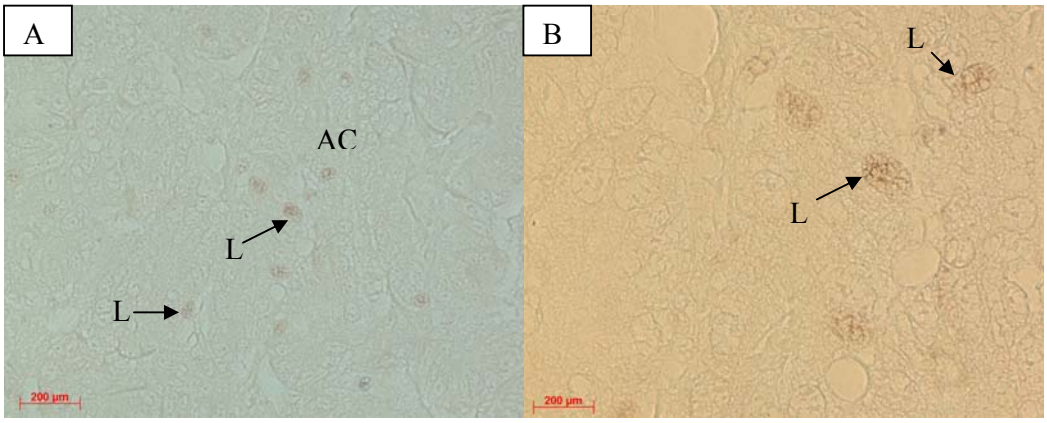


Figure 3.67A, B: Bcl-2 localisation (L) in moderately differentiated adenocarcinoma (AC) in muscularis propria (A = x400; B = x1000). Under higher magnification, this localisation can be identified as nuclear (B). Once again neither DWNN mRNA nor DWNN protein was upregulated in these areas therefore there is no correlation.

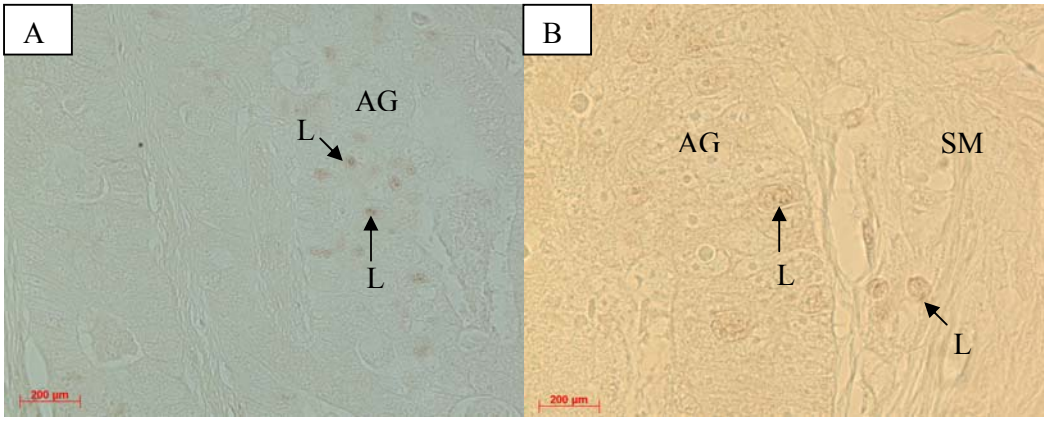


Figure 3.68A, B: Nuclear localisation of Bcl-2 in moderately differentiated adenomatous glands (AG) in submucosa (SM) (A = x400, B = x1000).

3.7.2 Summary

Suppression of apoptosis is seen in dysplastic zones of the tissues and this is to be expected, as apoptosis inhibition is one of the key factors promoting cancer progression. However, it is important to note that although Bcl-2 is localised in the vicinity of the DWNN mRNA and protein, at the particular areas where DWNN shows upregulation (from ISH and ICC) Bcl-2 is down-regulated and vice versa. In fact, from TUNEL studies it was verified that apoptosis was upregulated in these areas of DWNN upregulation therefore the gene is proposed to be pro-apoptotic.

3.8 *Helicobacter pylori* localisation

A number of investigations have identified the correlation between *Helicobacter pylori* infection and colon cancer. The mechanism for this is thought to be that *H. pylori* infection raises gastrin levels, which decrease the threshold for colon cancer development (Noshirwaim *et al.* 2000). Using antibodies raised against *H. pylori*, immunocytochemistry was carried out to localise *H. pylori* protein in normal and diseased tissue to establish whether this relationship holds true and how it relates to apoptosis occurrence. It was also done to establish whether any relationship could be found with DWNN expression.

3.8.1 *H. pylori* ICC images

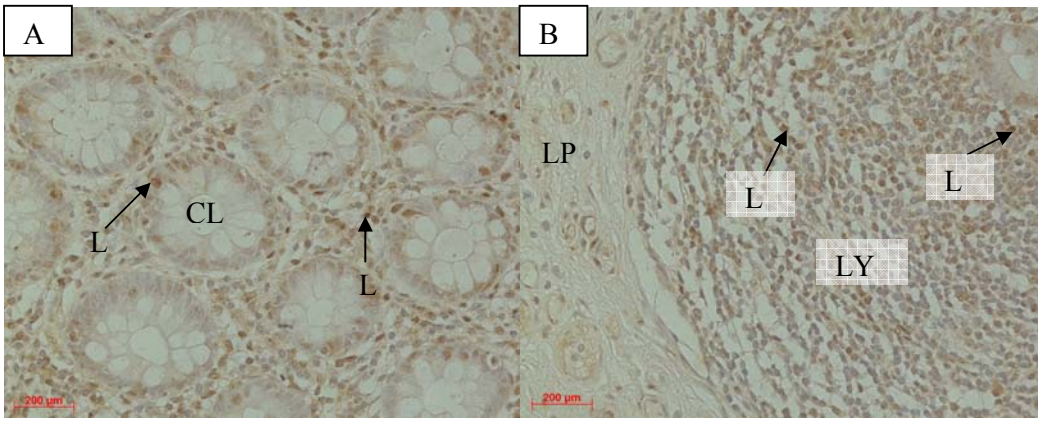


Figure 3.69A, B: Normal colon tissue showing abundant localisation (L) of *H. pylori* protein in lamina propria (LP) in between crypts of Lieberkühn (CL) in A, and localisation in lymphocytes (LY) adjacent to this area in B (A, B = x400). Although this case has no signs of cancer precursors, this test for the bacteria has given a positive result.

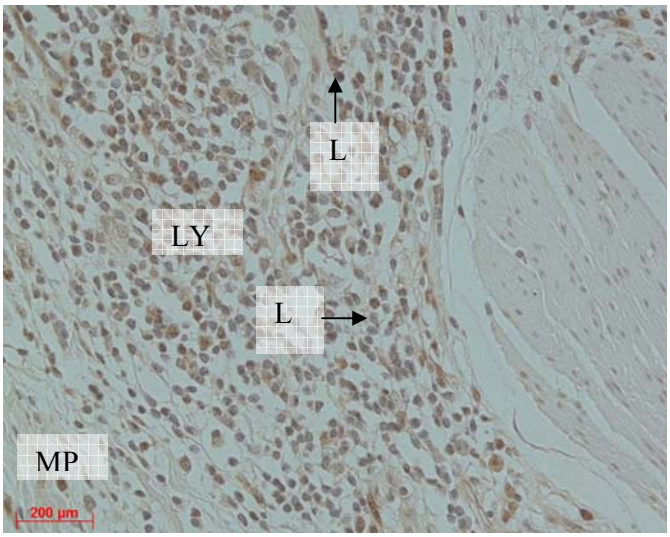


Figure 3.70: Localisation (L) in lymphocytes (LY) in muscularis propria (MP) adjacent to adenocarcinoma (x400).

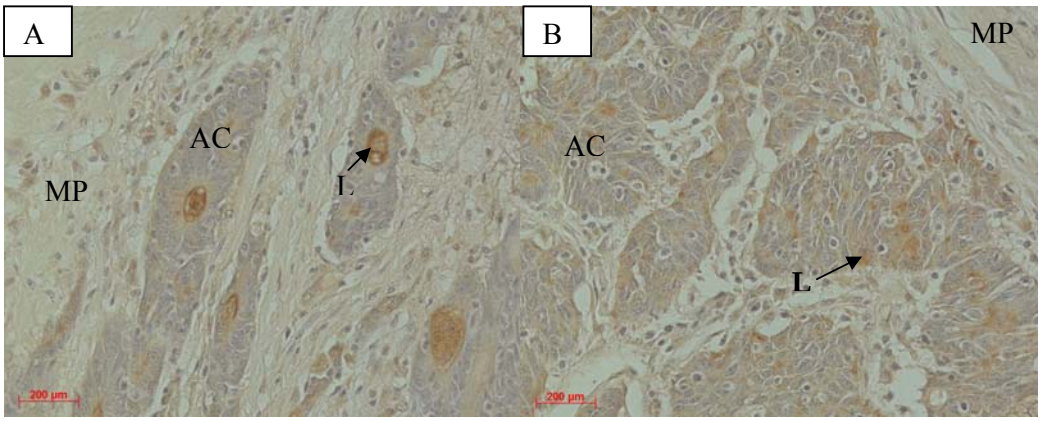


Figure 3.71A, B: *H. pylori* localisation (L) in poorly differentiated adenocarcinoma (AC) in muscularis propria (MP) (A, B = x400).

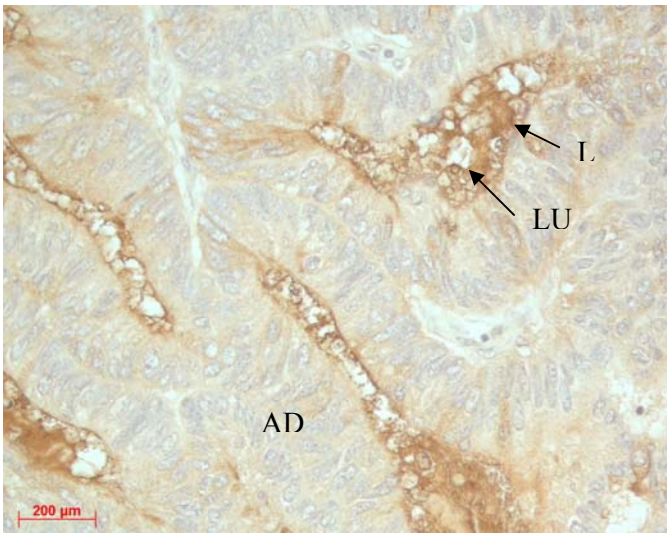


Figure 3.72: Cytoplasmic localisation (L) in lumen (LU) of moderately differentiated adenocarcinoma (AD) in muscularis propria (x400).

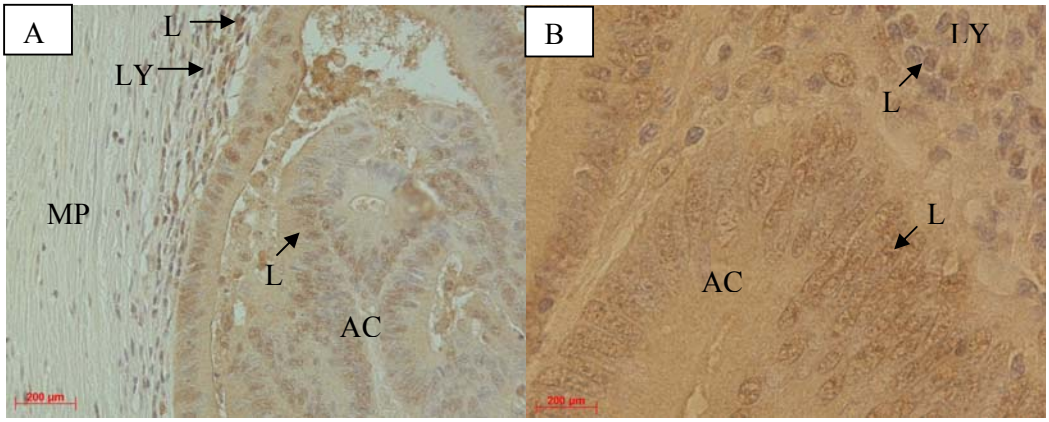


Figure 3.73A, B: Localisation (L) of *H. pylori* in well differentiated adenocarcinoma (AC) in the muscularis propria (MP) and in adjacent lymphocytes (LY) (A = x400; B = x1000).

3.8.2 Summary

The group 1 carcinogenic bacteria, *Helicobacter pylori*, cause cancer via the initiation of chronic inflammation, which may result in lifelong inflammation (Mahady *et al.*, 2002; Parsonnet, 1995). Previous studies have shown a high percentage of control cases, a greater percentage of patients with colonic polyps and an even greater percentage of colon cancer patients testing positively for the bacteria (Meucci *et al.*, 1997; Breuer-Katschinski *et al.*, 1999). Similarly, the normal colon tissue in this study showed significant localisation of *H. pylori,* yet there were no signs of polyps. In the diseased cases, localisation was found in all three grades of the adenocarcinomas and in adjacent lymphocytes.