3 CHAPTER 3: RESULTS

3.1 Histopathology

3.1.1 Normal Squamous Epithelium

The squamous epithelium that covers the ectocervix of the uterus is composed of different layers starting at the basement membrane (basal, parabasal, intermediate, superficial) (Figure 3.1). The basal cervical epithelial layers have the capacity to differentiate into multiple phenotypes. Clear cytoplasm results from too much glycogen content in the cell and this is under the influence of oestrogen. As the cells mature, the nuclei get smaller and the amount of cytoplasm increases.

3.1.2 Squamous cell carcinoma

3.1.2.1 Epidermoid cervical carcinoma

This is a poorly-differentiated squamous cell carcinoma. The cells are more loosely arranged and in irregular networks (figure 3.2). Stratified cells are large and rounded containing nuclei frequently of large size. The fibro-vascular stroma is sometimes abundant but sometimes scarce; it may contain inflammatory cells, including polymorphonuclear leucocytes which invade degenerating tumour cells.

3.1.2.2 Verrucous carcinoma

This is a well-differentiated squamous cell carcinoma with few mitoses (Figure 3.3). There are papillary fronds covered by large cells with eosinophilic cytoplasm. The deep margin is well demarcated and appears to push tissue aside rather than invading it.
3.1.2.3 Carcinoma in situ

This condition results from the proliferation of small cells with basophilic cytoplasm and disproportionately large nuclei. The cells are crowded together and the axes of the nuclei often lie perpendicular to the surface (Figure 3.4). There is disorganization and loss of the normal cellular stratification and replacement of the entire thickness of the epithelium by the abnormal cells. The transition from normal stratified epithelium to carcinoma in situ may be sudden as it leaves a demarcation line. There is a sharp distinction between the abnormal proliferation and the stroma of the cervix.
Figure 3.1: H&E of a normal epithelium.
The ectocervix composed of different layers starting at the basement membrane (basal (B), parabasal (P), intermediate (I), superficial (S)). Haematoxylin and eosin staining of the ectocervix with magnification 400X.

Figure 3.2: H&E of Epidermoid cervical cancer.
Epidermoid cervical cancer, showing abundant fibro vascular stroma (S), inflammatory infiltration of leucocytes (L) and irregular stratified cells (SC), magnification 400X.
Figure 3.3: H&E of verrucous carcinoma.
This is a well-differentiated squamous cell carcinoma with few mitoses (M) and papillary fronds (PF), magnification 400X.

Figure 3.4: H&E of Carcinoma in situ.
Carcinoma in situ showing proliferation of small cells with basophilic cytoplasm and disproportionately large nucleus (N), magnification 400X.
3.2 DWNN mRNA Expression Pattern in Human Cervical Cancer.

3.2 Introduction.

Pro-apoptotic activity of DWNN in human cervical cancer was elucidated by establishing its expression levels in this type of tumour. The aim of the study was to synthesize a probe which was used to detect expression levels of DWNN mRNA in human cervical cancer. RNA was extracted from normal kidney cell lines then reverse transcribed into cDNA, which was then used for PCR amplification which was further cloned into pGem-T-Easy and then labelled with Digoxigenin. The labelled probe was used to hybridize the appropriate sequence in the tissue section.

3.2.1 RNA Extraction

Total RNA was extracted from normal kidney cell lines (section 2.2.1.1.1) and analysed by electrophoresis on formaldehyde agarose gel. Intact total RNA run on a denaturing gel showed sharp, clear 28S and 18S rRNA bands (Figure 3.5). The 28S rRNA band was approximately twice as intense as the 18S rRNA band (Figure 3.5, lane 2). This 2:1 ratio (28S:18S) is a good indication that the RNA is completely intact.

3.2.2 Reverse Transcription (RT) - Analysis

RNA obtained from above was reverse transcribed to cDNA (section 2.2.1.2) and ran on agarose gel (Figure 3.6). Compared to the positive control RNA template (lane 4) there was a band shift from the molecular weight RNA to the higher molecular weight cDNA.
3.2.3 PCR amplification of DWNN gene sequence

PCR primers were designed around 5’1.1 kb and exon 16 of the DWNN gene sequence (Appendix 3). cDNA synthesized from the above was used as the template for PCR amplification. This PCR generated a ~200bp (5’1.1 kb) and ~100bp (exon 16).

3.2.4 Cloning of DWNN (3’6.1 kb, 3’1.1 kb, 5’1.1 kb and Exon 16) sequences into pGem-T-Easy

The DWNN variants fragments were cloned into pGem-T-Easy vector (section 2.2.1.4) (Appendix). Colony PCR was used to screen for positive clones (section 2.2.1.4.2) generated fragments of about 150bp, 200bp, 180bp and 100bp, suggesting all the colonies screened positive. Plasmid DNA isolation was performed on all the clones (section 2.2.1.5). The plasmid DNA was digested with EcoR1 and analysed by electrophoresis (figure 3.8) which confirms the presence of (3’6.1 kb), ~200bp (5’1.1 kb), ~300bp (3’1.1 kb) and ~100bp (exon 16).
Figure 3.5: Extracted RNA.

Lane 2 shows the intact RNA; the 18S and 28S ribosomal RNA bands are clearly visible with no contamination.

Figure 3.6: Reverse transcription.

Lane 1: 100 bp marker, Lane 2: cDNA from Oligo dT primers, lane 3: cDNA duplicate, lane 4: positive control supplied with the kit, lane 5: negative control.
Figure 3.7: Polymerase chain reaction
Lane 1: 100bp marker, lane 2: negative control, lane 3: 5’1.1 kb and lane 4: exon 16.

Figure 3.8: Agarose gel electrophoresis of restriction digests.
Lane 1: molecular weight marker; Lane 2: Exon 16 EcoR1; Lane 3: Exon 16 Pst 1; Lane 4: 5’1.1 kb Pst; Lane 5: 5’1.1 kb EcoR1; Lane 6: 3’1.1 kb EcoR1; Lane 7: 3’1.1 kb Pst 1; Lane 8: 3’6.1 kb EcoR1 and Lane 9: 3’ 6.1 kb Pst 1.
3.2.5 In situ hybridization

This is a method of localising either mRNA within cytoplasm or DNA within the chromosomes of the nucleus, by hybridizing the sequence of interest to a complementary strand of a nucleotide probe. The basic principle is to use the probe to detect specific nucleotide sequences within cells and tissues.

The RNA probes are usually achieved by in vitro transcription from a linearized DNA template (clone) which contains a promoter for RNA polymerase (section 2.2.1.9); the advantage of this probe is that it can be detected with antibodies conjugated to a number of different enzymes such as alkaline phosphatase, which deposits a dark purple precipitate in the tissues, which then turns deep blue colour after exposure to ethanol when incubated in the presence of the substrate NBT/BCIP. Anti-DIG antibody is used to
visualize the probe. They also have the advantage that RNA-RNA hybrids are very thermostable and are resistant to digestion by RNases, in contrast to DNA probes they are less sensitive because of the tendency of the DNA strands to rehybridize to each other.

Figure 3.11: Schematic representation of a DIG-labelling reaction.

Figure 3.12 (A) represents a negative control whereby the probe was replaced with hybridization buffer. There were very low levels of the 5’ 1.1 kb mRNA probe in a normal tissue section (B) but a moderately differentiated carcinoma and its surrounding stroma (C) showed elevated levels of the probe. In moderately-differentiated carcinoma (D) also showed high levels of expression. In the normal stroma (figure 3.13), the 5’1.1 kb probe was found to localize in the mesonephric ducts (E) and the infiltrating lymphocytes (F). The 5’ 1.1 kb mRNA probe was found to localize both in the cytoplasm and nucleus, but the normal tissue section showed little nuclear staining. Figure 3.14 indicated low levels of 5’ 1.1 kb probe in the cytoplasm of well-differentiated carcinoma.
In a normal section (figure 3.15 B) low levels of 3’ 1.1 kb mRNA were observed. A moderately differentiated carcinoma (C,D) showed high expressions of the probe; the invaded stroma also showed high levels of 3’ 1.1 kb mRNA. The smooth muscle cells (figure 3.16E) and the fibrous stroma (figure 3.16F) also showed high expression of the probe. A well-differentiated carcinoma (figure 3.17) showed low levels of expression. The 3’ 1.1 kb mRNA was localised both in the cytoplasm and nucleus.

The 3’ 6.1 kb mRNA probe was found in low levels in the normal section (figure 3.18 B), in contrast moderately differentiated carcinoma showed high levels of 3’ 6.1 kb mRNA (C, D). The invaded stroma also indicated high expression of the probe. The probe was localized in the nucleus. The probe was highly expressed in the fibrous stroma (figure 3.19E, F). Well-differentiated carcinoma (figure 3.20G,H) showed very low levels of the probe and only cytoplasmic localization was observed.

Figure 3.21 illustrates low levels of exon 16 in a normal section (B). In contrast, moderately differentiated carcinoma (C, D) showed high expression levels of exon 16, both in the nucleus and cytoplasm. A well-differentiated carcinoma showed very low level of exon 16 expression.

Figure 3.22 shows fluorescent in situ hybridization, (A) indicates a negative control whereby a probe was replaced with hybridization buffer and (B) shows the low levels of expression of the 5’1.1 kb probe in a normal tissue section.
There were elevated levels of the 3’ 1.1 kb mRNA in moderately differentiated carcinoma and the stroma (figure 3.23 A) but low levels of expression were observed in well differentiated carcinoma (B).

Figure 3.24 (A) shows high expression of 3’ 6.1 kb mRNA in moderately differentiated carcinoma and the surrounding stroma; in contrast, low levels were shown in well differentiated carcinoma (B).

High expression levels of exon 16 were observed in moderately differentiated carcinoma (figure 3.25, A) but low levels in the well differentiated carcinoma (B).

### 3.2.6 Summary

A probe was produced by the inclusion of the sequence of interest in *E.Coli* which was replicated, lysed and the DNA extracted, purified and the sequence of interest was excised by EcoR1 restriction enzyme. The sequence was confirmed and appropriate primers were designed to produce the relevant sequence very rapidly by PCR. The advantage of the bacterial preparation is that it is possible to obtain large quantities of the sequence of interest. In vitro transcription of linearized plasmid DNA with RNA polymerase was used to produce the RNA probes. The plasmid vector containing polymerases from bacteriophages T7 and sp6 was used. T7 polymerase synthesised the antisense strand, whilst sp6 synthesised the sense strand. DWNN was found to be more
highly expressed in moderately differentiated carcinoma than well-differentiated carcinoma. This gene was localized both in the cytoplasm and nucleus.

Figure 3.12: In situ hybridization of 5’ 1.1 kb probe in moderately-differentiated carcinoma.

(A) indicates a negative control and (B) shows low levels of 5’ 1.1 kb probe in the stroma of a normal section. (C) illustrates high expression in moderately differentiated carcinoma and the surrounding stroma and (D) also shows elevated levels in moderately-differentiated carcinoma. The section was counterstained with haematoxylin and detected using NBT/BCIP, magnification 400X (B, C) and 1000X (A, D).
Figure 3.13: In situ hybridization of 5’ 1.1 kb probe in the stroma.

(E) represents high expression of 5’1.1 kb in the mesonephric ducts in the stroma and infiltrating lymphocytes in the stroma (F). The sections were counterstained with haematoxylin and detected using NBT/BCIP, magnification 1000X.

Figure 3.14: In situ hybridization of 5’ 1.1 kb probe in well-differentiated carcinoma.

This indicates low levels of 5’1.1 in the cytoplasm of well differentiated carcinoma (G, H). The section was counterstained with haematoxylin and detected using NBT/BCIP, magnification 1000X.
Figure 3.15: In situ hybridization of 3’ 1.1 kb probe in moderately-differentiated carcinoma.

(A) indicates a negative control and (B) shows low levels of 3’ 1.1 kb probe in the stroma of a normal section. Figure 3.15 (C) illustrates high expression in moderately differentiated carcinoma and (D) showed elevated levels in the cytoplasm of moderately-differentiated carcinoma. The section was counterstained with haematoxylin and detected using NBT/BCIP, magnification 400X (A) and 1000X (B, C, and D).
Figure 3.16: In situ hybridization of 3’ 1.1 kb probe in the stroma.

The 3’ 1.1 kb is elevated in the nucleus and cytoplasm of the cells found within the smooth muscles (E) and fibrous stroma (F). The section was counterstained with haematoxylin and detected using NBT/BICP, magnification 1000X.

Figure 3.17: In situ hybridization of 3’1.1 kb probe in well-differentiated carcinoma.

The figure illustrates low cytoplasmic staining in the cytoplasm of well-differentiated carcinoma cells (F,G). The section was counterstained with haematoxylin and detected using NBT/BICP, magnification 1000X.
Figure 3.18: In situ hybridization for 3’ 6.1 kb probe in moderately differentiated carcinoma.

(A) indicates a negative control and (B) shows low levels of 3’ 6.1 kb probe in the stroma of a normal section. (C) illustrates high expression in moderately differentiated carcinoma and the surrounding stroma and (D) showed low levels in well-differentiated carcinoma. The section was counterstained with haematoxylin and detected using NBT/BCIP, magnification 400X (A) and 1000X (B, C, and D).
Figure 3.19: In situ hybridization of 3’6.1 kb probe in the stroma.
This diagram shows high levels of 3’ 6.1 kb in the fibrous stroma (E) and infiltrating lymphocytes in the stroma (F). The section was counterstained with haematoxylin and detected using NBT/BCIP, magnification 1000X.

Figure 3.20: In situ hybridization of 3’6.1 kb probe in well-differentiated carcinoma.
This shows low expressions of 3’ 6.1 kb probe in the cytoplasm (G, H). The section was counterstained with haematoxylin and detected using NBT/BCIP, magnification 1000X.
Figure 3.21: In situ hybridization for exon 16 probe in moderately-differentiated carcinoma.

(A) indicates a negative control and (B) shows low levels of exon 16 in the stroma of a normal section. (C) illustrates high expression in moderately differentiated carcinoma and the surrounding stroma and (D) showed high levels in moderately-differentiated carcinoma. The section was counterstained with haematoxylin and detected using NBT/BCIP, magnification 400X (A) and 1000X (B, C and D),
Figure 3.22: Fluorescent in situ hybridization of 5’ 1.1 kb probe.
This illustrates a negative control whereby a probe was replaced with hybridization buffer (A), and a normal tissue sections (B) shows low expression levels of the probe (3’ 6.1 kb). This was detected using FITC, magnification 400X.

Figure 3.23: Fluorescent in situ hybridization of 3’1.1 kb probe.
(A) illustrates high expression of 3’1.1 kb in moderately differentiated carcinoma and the surrounding stroma and (B) shows low levels in well-differentiated carcinoma. FITC was used for detection, magnification 400X.
Figure 3.24: Fluorescent in situ hybridization of 3’6.1 kb probe

(A) represents elevated levels of 3’6.1 kb in the invaded stroma and moderately differentiated carcinoma, and (B) shows low levels in well-differentiated carcinoma. This was detected using FITC, magnification 400X (A) and 1000X (B).

Figure 3.25: Fluorescent In Situ Hybridization of Exon 16.

(A) shows high expression levels of exon 16 in the invaded stroma of moderately differentiated carcinoma and (B) shows low expression levels of exon 16 in well-differentiated carcinoma. This was detected using FITC, magnification 1000X.
3.3 Tissue Distribution of the DWNN Protein in Human Cervical Cancer

3.3.1 Introduction

Immunocytochemistry helps in the diagnosis of many pathologic conditions in which tissue is obtained at surgery, from biopsy, via cytological sampling or at autopsy. It uses an antigen-antibody reaction coupled with a reaction that produces a chromogen (coloured product) to identify specific components in tissues. The markers attached to the secondary antibody can be viewed by light microscopy. The chromogen is produced via reaction with peroxidase. The peroxidase used is horseradish peroxidase, and it has to be attached to the secondary antibody and it is the localization of this peroxidase in the tissues that provides the ability to identify specific components. The peroxidase must act upon a compound to produce the chromogen. Most often, the compound used is 3’3 diaminobenzidine tetrachloride (DAB). A visible insoluble dark-brown precipitate is formed by DAB in the presence of peroxidase. In this work, DWNN protein expression pattern was confirmed with the levels of DWNN mRNA, to establish its expression levels in human cervical cancer. Bcl-2 expression was also done to confirm which apoptotic cascade or apoptotic components take part in the development of cervical cancer, and its expression was compared with that of DWNN.
3.3.2 Antibody profiles

3.3.2.1 Anti-DWNN antibody

The primary antibodies, namely antihuman DWNN antibodies, were raised against the DWNN domain in the rabbit. The DWNN antibody used in this study was obtained from University of the Western Cape. It was raised in rabbit against the short form DWNN (13kDa).

<table>
<thead>
<tr>
<th>MSCVHYKFSKLNYYDTVTFDGLHISLCDLKQIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GREKLAADCQLQITNAQTKEETYTDNALIPKNS</td>
</tr>
<tr>
<td>SVIYRRIPIGGVKSSTKTYVISRTPEAMATTKAVC</td>
</tr>
<tr>
<td>KNTISFFYTLLLPL</td>
</tr>
</tbody>
</table>

Figure 3.26: Amino acid sequence of DWNN (13 kDa) to which antibodies were raised against.

3.3.2.2 Anti-Bcl-2 antibody

The Bcl-2 family of proteins plays an important role in the regulation of cell death. Human Bcl-2 is a 26kDa, membrane integrated, anti-apoptotic oncoprotein. It interacts with other apoptotic proteins (Bax, Bad, Bid, Bik, Bak) to promote cell survival (Yang et al., 1997). Mutations in Bcl-2 gene results in the development of cancer.

Bcl-2 is a monoclonal IgG1 antibody which was raised against a recombinant protein corresponding to amino acids 1-205 of the human origin (Santa Cruz Biotechnology).
3.3.3 Immunocytochemical localisation of DWNN protein

3.3.3.1 DWNN-13 kDa

Immunocytochemistry allows phenotypic markers to be detected and interpreted within a morphologic context. This technique allows cell specific localization of proteins in tissue sections, using antibodies directed against specific antigen peptide sequence. Peroxidase is an enzyme conjugate which revealed the localization of antibody-bound antigenic sites by means of a coloured reaction. DAB is the chromogenic substrate for peroxidase, which stained antigen-antibody sites brown. The protein labelling in the tissue section was detected using DAB chromogen system which gave out a brown stain. Tissue sections were incubated with the primary antibody (anti-DWNN) as described in section 2.3.3.1.

![Diagram of immunocytochemistry reaction](image)

Figure 3.27: Schematic representation of immunocytochemistry reaction.
Figure 3.28 B, C illustrates elevated levels of DWNN protein in moderately-differentiated carcinoma; in contrast, figure 3.29E and F showed low levels of DWNN in well-differentiated carcinoma.

Figure 3.30H demonstrates strongly detected immunocytochemical staining of DWNN in metaplastic epithelium. The parabasal layer of a normal section showed some nuclear and cytoplasmic DWNN immunoreactivity, whilst the intermediate layer showed no immunoreactivity, but there was also some positive staining on the superficial layer; this is illustrated in figure 3.30I. Positive immunoreactivity was weakly detected in the normal endocervical glands, as in figure 3.31K. Figure 3.31L shows strong immunoreactivity in the cytoplasm and nuclei of the dysplastic endocervical glands. There was also some nuclear staining of the columnar cells that line the endocervical glands in figure 3.31 K.

Figure 3.32N demonstrates a strong positive cytoplasmic staining of the periphery of fibroblasts in the stroma of cervical cancer tissue sections. The keratin pearls showed strong staining and this is demonstrated in figure 3.32O. The mesonephric ducts in the stroma were positively stained in figure 3.33Q, which also shows positive immunoreactivity in the cytoplasm of the tunnel cluster (R).
3.3.3.2 DWNN-200 kDa

Figure 3.35 A and B showed high expression of DWNN-200kDa protein immunoreactivity in moderately-differentiated carcinoma, also figure 3.36 C,D indicated low levels of DWNN protein in well-differentiated carcinoma. Nuclear and cytoplasmic staining was observed.

3.3.3.3 Localisation of Bcl-2 protein

Bcl-2 is a proto-oncogene situated in the inner mitochondrial membrane. It is a 24kDa protein with 239 amino acids which protects the cells from apoptosis and is localised in the long arm of the 18th chromosome. It is found in the endoplasmic reticulum and some parts of the nuclear membrane. To give an idea of which apoptotic cascade or apoptotic components take part in the development of cervical cancer, the localisation of the anti-apoptotic Bcl-2 protein was done (section 2.3.3.1) and it was mostly observed in the cytoplasm and to a lesser extent in the nucleus (Figure 3.37).

In normal tissue sections the basal layer showed no staining; only the intermediate layer showed some cytoplasmic staining (figure 3.37B), whereas the superficial layer showed cytoplasmic immunoreactivity. Dysplastic epithelium showed strong positivity, both cytoplasmic and nuclear, as in figure 3.37A. The smooth muscle cells showed some nuclear staining. The dysplastic endocervical glands showed immunoreactivity only in the cytoplasm 3.37D and normal endocervical glands stained positive in the nuclei (figure 3.37C)
Figure 3.38B demonstrates some cytoplasmic and a small amount of nuclear positive staining in invasive carcinoma. Well-differentiated squamous carcinoma showed cytoplasmic staining (figure 3.38A). In the stroma, some lymphocyte infiltrates stained positive. The squamous pearls showed immunoreactivity in the cytoplasm (Figure 3.38C). The keratin pearls showed cytoplasmic staining (figure 3.38D). Some of the invasive carcinomas showed immunoreactivity in the cytoplasm and the membrane. Squamous carcinoma showed staining within the cytoplasm and along the membranes. The mesonephric ducts also showed nuclear and cytoplasmic immunoreactivity. Not illustrated.
Table 3.1: Summary of DWNN labelling results showing intensity of labelling in the different cell types and whether nuclear or cytoplasmic.

<table>
<thead>
<tr>
<th>Different cell type</th>
<th>Intensity of labelling</th>
<th>Cytoplasmic or nuclear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Islands of tumour</td>
<td>+++</td>
<td>Cytoplasmic and nuclear</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>+++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Tunnel clusters</td>
<td>+++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Keratin pearls</td>
<td>+++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Dysplastic endocervical glands</td>
<td>++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Carcinoma in situ</td>
<td>+++</td>
<td>Cytoplasmic and nuclear</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>+++</td>
<td>Cytoplasmic and nuclear</td>
</tr>
<tr>
<td>Poorly differentiated squamous cell carcinoma</td>
<td>+++</td>
<td>Cytoplasmic and nuclear</td>
</tr>
<tr>
<td>Moderately-differentiated squamous cell carcinoma</td>
<td>+++</td>
<td>Cytoplasmic and nuclear</td>
</tr>
<tr>
<td>Well-differentiated squamous cell carcinoma</td>
<td>++</td>
<td>Cytoplasmic and nuclear</td>
</tr>
<tr>
<td>Normal stroma</td>
<td>++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Normal endocervical glands</td>
<td>++</td>
<td>Cytoplasmic and nuclear</td>
</tr>
<tr>
<td>Normal epithelium</td>
<td>++</td>
<td>Cytoplasmic and nuclear</td>
</tr>
</tbody>
</table>

+++ - High intensity
++ - Low intensity
Table 3.2: Summary of Bcl-2 labelling results showing intensity of labelling in the different cell types and whether nuclear or cytoplasmic.

<table>
<thead>
<tr>
<th>Different cell type</th>
<th>Intensity of labelling</th>
<th>Cytoplasmic or nuclear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal epithelium</td>
<td>+++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Dysplastic epithelium</td>
<td>+++</td>
<td>Cytoplasmic and nuclear</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td></td>
<td>Nuclear</td>
</tr>
<tr>
<td>Normal endocervical glands</td>
<td>+++</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Dysplastic endocervical glands</td>
<td>++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Moderately-differentiated carcinoma</td>
<td>++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Well-differentiated carcinoma</td>
<td>+++</td>
<td>Cytoplasmic and nuclear</td>
</tr>
<tr>
<td>Keratin pearls</td>
<td>+++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Invaded stroma</td>
<td>++</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Tunnel cluster</td>
<td>++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Mesonephric ducts</td>
<td>++</td>
<td>Cytoplasmic and nuclear</td>
</tr>
</tbody>
</table>

3.3.4 Summary

The anti-human DWNN antibody was raised in rabbits at University of Western Cape. This was used for immunocytochemistry of DWNN protein, to assess its expression levels in cervical cancer. DWNN was expressed in the cells of the superficial, suprabasal, basal layers but not in intermediate cells. In metaplastic cervical epithelium DWNN staining was found throughout the epithelium. In squamous carcinoma, islands of tumour, metaplastic endocervical glands and invasive squamous carcinoma, there was strong DWNN immunostaining. Bcl-2 immunostaining was found throughout the cytoplasm with low concentrations in the normal tissue sections. There was low intensity of immunostaining in cervical lesions. High intensity labelling indicates high expression of the protein. In contrast, there were low expression levels of the anti-apoptotic Bcl-2 protein at the areas where DWNN was highly expressed. Up regulation of DWNN was
observed in dysplastic epithelium, but it was down-regulated only in the intermediate and basal layers of normal epithelium and in normal endocervical glands. It was also up-regulated in hyperplastic endocervical glands, islands of tumour, tunnel clusters, mesonephric rests and in the keratin pearls. It was observed to be highly expressed in cancerous sections and down-regulated in normal sections. Bcl-2 was up-regulated in most normal sections, also in the islands of tumour, but down-regulated in cancerous sections.

3.4 Image Analysis

A digital image consisted of pixels, each with its own value on a grey scale between 1 and 256. The values of pixels which represent immunolabelling (those with a brown colour - DAB) have been shown to fall within a threshold of 160 to 256. In order to achieve a higher n value, the areas of interest were divided into 3 to 4 regions. For each area a separate set of results was collected; the area of each analysed region of interest (um²). The grey scale (1-256) was divided into 8 phases, each with its own upper and lower threshold. The phases 6 - 8 represent high immunolabelling, so these are the areas of interest. The total numbers of pixels that fall within these phases were calculated.

In order to get a unit that can be used across all the images, the total number of pixels that represent immunolabelling (phases 6-8) were calculated per area, so the unit that was used is pixels / um².
Figure 3.34 indicates high expression value of DWNN in the islands of tumour, mesonephric rests, dysplastic epithelium as compared to a normal tissue section which has low expression values of the protein. Negative control was done to compare the background. The error bars on the graph indicates the difference in DWNN staining.

Figure 3.28: Immunocytochemistry of DWNN-13 kDa in the moderately-differentiated squamous cell carcinoma.

(B) indicates high intensity of DWNN immunostaining in the cytoplasm and the nuclei of the carcinoma cells; also (C) represent immunoreactivity of DWNN in the carcinoma cells of the moderately differentiated tumour. Labelling was counterstained with haematoxylin and detected with DAB chromogen, magnification 200X (A) and 400X (B, C).
Figure 3.29: Immucytochemistry of 13 kDa DWNN in well-differentiated squamous cell carcinoma.

(E, F) represents cytoplasmic accumulation of DWNN in the well differentiated carcinoma cells. Labelling was counterstained with haematoxylin and detected with DAB chromogen, magnification 200X (D) and 400X (E, F).

Figure 3.30: Immunocytochemistry of DWNN-13 kDa on the epithelium.

(H) Illustrates high intensity of DWNN immunostaining in a dysplastic epithelium and (I) represents immunostaining of a normal tissue section in the superficial, parabasal layers; the intermediate and basal layers showed negative labelling. Labelling was counterstained with haematoxylin and detected with DAB chromogen, magnification 200X (G) and 400X (H, I).
Figure 3.31: Immunocytochemistry of DWNN-13 kDa on the endocervical glands. (K) Represents nuclear staining in the normal endocervical glands and (L) indicates intense staining of the dysplastic endocervical glands. Labelling was counterstained with haematoxylin and detected with DAB chromogen, magnification 200X (J) and 400X (K, L).

Figure 3.32: Immunocytochemistry of DWNN-13 kDa on the fibroblasts and keratin pearls. (N) and (O) show strong positive DWNN immunostaining in the fibroblasts and keratin pearls. Labelling was counterstained with haematoxylin and detected with DAB chromogen, magnification 200X (M) and 400X (N, O).
Figure 3.33: Immunocytochemistry of DWNN-13 kDa on tunnel cluster and mesonephric ducts.
(Q, R) indicates DWNN labelling on the tunnel cluster (TC) and mesonephric ducts (MD) of the cervical cancer respectively. Localization was mostly in the cytoplasm. Labelling was counterstained with haematoxylin and detected with DAB chromogen, magnification 200X (P) and 400X (Q, R).

Figure 3.34: DWNN image analysis
Representation of immunolabelled DWNN, indicating high expression of DWNN protein in the cells of a cancerous section in contrast to a normal tissue section.
Table 3.3: The results of an ANOVA statistical test

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Df</th>
<th>Mean Squares</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between</td>
<td>3775.0</td>
<td>13</td>
<td>290.4</td>
<td>12</td>
</tr>
<tr>
<td>Error</td>
<td>2081.1</td>
<td>86</td>
<td>24.20</td>
<td></td>
</tr>
<tr>
<td>total</td>
<td>5856.1</td>
<td>99</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The probability of this result, assuming the null hypothesis, is 0.000

Table 3.4: The results of Kruskal-Wallis statistical test

<table>
<thead>
<tr>
<th>H (observed value)</th>
<th>75.332</th>
</tr>
</thead>
<tbody>
<tr>
<td>H (critical value)</td>
<td>23.685</td>
</tr>
<tr>
<td>Df</td>
<td>14</td>
</tr>
<tr>
<td>One-tailed p-value</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Alpha</td>
<td>0.05</td>
</tr>
</tbody>
</table>

At the level of significance Alpha=0.050 the decision is to reject the null hypothesis of absence of difference between the 15 samples. In other words, the difference between the samples is significant.

Figure 3.35: Immunocytochemistry of DWNN-200 kDa on moderately differentiated squamous cell carcinoma.

This figure indicates cytoplasmic labelling of DWNN 200kDa protein in moderately differentiated squamous carcinoma cells. Labelling was counterstained with haematoxylin and detected with DAB chromogen, magnification 400X (A) and1000X (B).
Figure 3.36: Immunocytochemistry of DWNN-200 kDa on well-differentiated squamous cell carcinoma.

(C, D) illustrates weak staining of DWNN 200 kDa protein in the nucleus and cytoplasm of well-differentiated carcinoma cells. Labelling was counterstained with haematoxylin and detected with DAB chromogen, magnification 400X.
Figure 3.37: Immunocytochemistry of Bcl-2 on endocervical glands.

Illustration of Bcl-2 expression in cervical cancer (A), thus the dysplastic epithelium. B shows specific labelling at the different layers of the normal epithelium. C and D show labelling of the normal endocervical glands and dysplastic endocervical glands respectively. Labelling was counterstained with haematoxylin and detected with DAB chromogen, magnification 400X.
Figure 3.38: Immunocytochemistry of Bcl-2 on squamous cell carcinoma.

Illustration of Bcl-2 expression in cervical cancer. A and B show labelling of the moderately-differentiated carcinoma and well-differentiated squamous carcinoma respectively. C indicates labelling of the squamous pearls and D represents labelling at the keratin pearls. Labelling was counterstained with haematoxylin and detected with DAB chromogen, magnification 400X.
3.5 Apoptosis Detection using TUNEL

3.5.1 Introduction

With increased understanding of the physiological events that occur during apoptosis, a number of assay methods have been developed for its detection. These assays are based on different events which characterise the apoptotic process. Apoptosis is characterised by fragmentation of the genomic DNA. The many breakpoints can be visualized with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) reaction. The enzyme terminal deoxynucleotidyl transferase (TdT) adds biotinylated nucleotides to the broken DNA ends. The biotinylated DNA can then be visualised by light microscopy.

3.5.2 Apoptotic activity

This method measures the fragmented DNA of apoptotic cells incorporating biotinylated nucleotide at the 3’ OH ends of the DNA fragments. Apoptotic cells detected were visualized as cells with dark bodies in the nucleus. Normal sections showed substantial levels of tunel reactive nuclei in figure 3.39B. There was a progressive decrease in the intensity of apoptosis as histological abnormality increased. The normal stroma showed increased nuclear staining especially within the smooth muscle cells, figure 3.39B. Figure 3.39C illustrates high apoptotic levels found in the invaded stroma. In well-differentiated squamous carcinoma, some of the nuclei showed staining; only the surrounding stroma indicated high apoptotic levels as in figure 3.39D. Some of the poorly differentiated carcinomas showed very few of the nuclei staining (figure 3.40E), and the dysplastic endocervical glands showed very marked decrease in apoptotic bodies (Figure
3.40F). Some of the invasive carcinomas also showed some nuclear positivity (figure 3.40G). Squamous nests also showed no staining, but the stroma was apoptotic (figure 3.40H).

**Table 3.5:** Summary of apoptosis labelling results showing intensity of labelling in the different cell types and whether nuclear or cytoplasmic.

<table>
<thead>
<tr>
<th>Different cell type</th>
<th>Intensity of labelling</th>
<th>Cytoplasmic or nuclear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal epithelium</td>
<td>+++</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>+++</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Dysplastic epithelium</td>
<td>+++</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Moderately differentiated carcinoma</td>
<td>+++</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Well-differentiated carcinoma</td>
<td>++</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Invaded stroma</td>
<td>+++</td>
<td>Nuclear</td>
</tr>
</tbody>
</table>

**3.5.3 Summary**

TUNEL is used as a marker for dying cells and apoptotic cells detected were visualised as cells with dark brown bodies in the nucleus. Normal cervical tissues showed substantial levels of TUNEL reactive nuclei in the smooth muscles and superficial layers of the epithelium. There were scattered apoptotic cells among the poorly-differentiated squamous carcinoma, well-differentiated squamous carcinoma cells and dysplastic cervical epithelium. There was a progressive decrease in the degree of apoptosis as the
histological gradation of malignancy increased. These cells were separated from the surrounding ones and show shrinkage of the nuclei and cytoplasm.

Figure 3.39: Apoptosis in normal and invaded stroma.

A shows no labelling in a negative and B indicates intense apoptosis positivity at the smooth muscles of a normal section. C and D illustrates high apoptotic index in the infected stroma of moderately-differentiated carcinoma and well-differentiated carcinoma respectively; labelling was detected using DAB chromogen, magnification 400X.
Figure 3.40: Apoptosis in endocervical glands and islands of tumour.

E and F also indicate no labelling in poorly differentiated squamous carcinoma and labelling in the dysplastic endocervical glands respectively. G and H show labelling of the islands of tumour and no labelling of the squamous nests but of the infected stroma; labelling was detected using DAB chromogen, magnification 400X.
3.6 Tumour proliferation assay using Ki67 antigen.

3.6.1 Introduction

Ki67 is an intracellular antigen expressed in proliferating cells (late G1-S phase, G2 and G0 phase). It is a bimolecular complex of 345kDa and 395kDa. The encoding gene is in chromosome 10 (10q25). Demonstration of ki67 expression indicates proliferative activity in tumours, thus it regulates cell proliferation. Avidin-biotin complex uses HRP-labelled streptavidin. The secondary antibody is conjugated with biotin and the streptavidin-HP complex reacts with biotin in the secondary antibodies. The resulting biotin-avidin-HP complex can react with the primary antibody bound to the specific epitope of the streptavidin complex, then catalyzes the substrate/chromogen reaction to form a coloured reaction (brown) using DAB as the substrate at the antigen site.

![Schematic representation of ABC staining reaction](image)

**Figure 3.41: Schematic representation of ABC staining reaction**
3.6.2 Localisation of ki67 protein

Demonstration of ki67 expression also indicates proliferative activity in tumours. In normal sections, there was decreased nuclear staining in the basal layer, and no staining was observed in the parabasal and intermediate layers, but there was cytoplasmic staining in the superficial layer (Figure 3.42A). Figure 3.42 B shows positive nuclear staining in the membranes of the metaplastic epithelium. Figure 3.42 C indicates weak labelling of the normal endocervical glands and figure 3.42D shows labelling of the invasive squamous cell carcinoma. In Figure 3.43 E&F the infected stroma showed nuclear staining only. Figure 3.43G shows labelling of the poorly differentiated squamous cell carcinoma and figure 3.43H indicates labelling of the well-differentiated carcinoma with strong staining of the keratin pearls.

<table>
<thead>
<tr>
<th>Different cell type</th>
<th>Intensity of labelling</th>
<th>Cytoplasmic or nuclear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal stroma</td>
<td>+++</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Normal endocervical glands</td>
<td>+++</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Keratin pearls</td>
<td>+++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Dysplastic epithelium</td>
<td>+++</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Moderately-differentiated carcinoma</td>
<td>+++</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Well-differentiated carcinoma</td>
<td>++</td>
<td>Cytoplasmic</td>
</tr>
</tbody>
</table>
3.6.3 Summary

The total proliferative status was assessed by the expression of ki67. Ki67 positive cells appeared in all the layers of the normal epithelium. In dysplastic epithelium indication of positive reaction is present in the thickness of the epithelium. In invasive squamous cell carcinoma, almost all cells of the surface epithelium and tumour islands in the underlying stroma were ki67 positive. Ki67-positive cells appeared in the infected stroma. There was an apparent increase in the proliferative status as the lesions progressed through increasing histological abnormality. The expression levels of DWNN and ki67 were both high. In areas where DWNN was highly expressed, increased proliferation of cells was also observed.
Figure 3.42: Ki67 expression on the epithelium, endocervical glands and islands of tumour.

A indicates ki67 positive cells in the nuclei of superficial cells of the basal layer and B shows labelling along the membranes of the dysplastic epithelium. C and D illustrate weak staining of the nuclei of the normal endocervical glands and invasive carcinoma respectively; labelling was detected by DAB chromogen, magnification 400X.
Figure 3.43: Ki67 expression on the invaded stroma and squamous cell carcinoma.

Representation of ki67 expression in cervical cancer. E and F show labelling of the infected stroma. H indicates labelling of the poorly differentiated carcinoma and I show labelling of a well differentiated carcinoma and intense staining of the keratin pearls; labelling was detected by DAB chromogen, magnification 400X.
3.7 LightCycler PCR: Confirms DWNN expression levels

A two-step RT-PCR method was adopted in this study so that the RT and the LightCycler PCR steps could be optimized for accurate quantification. The specificity and efficiency of each gene-specific LightCycler PCR was determined using the LightCycler melting curve analysis with SYBR Green I dye. Since SYBR Green I bind to double-stranded DNA at sequence-specific melting temperatures (Tm), each unique DNA species was identified based on its peak fluorescence at a specific temperature. MgCl₂ concentration and annealing temperatures of each LightCycler PCR were adjusted systematically until the melting curve analysis revealed a single fluorescent peak at the expected temperature and the formation of primer dimers, seen as a low and broad fluorescent peak at temperatures 10 to 15°C than that of the expected PCR product.

Figure 3.44 shows the melting curve analysis results for each gene-specific LightCycler PCR. Figure 3.44 (A) indicates a single melting curve of DWNN at 85°C and (B) and (C) represents a single peak of exon 16 and 3’6.1 kb at 79°C and 85°C respectively. The presence of a single peak at the expected temperature indicates that the primers are specific and non-specific amplification is not occurring.
Figure 3.44: Amplification curves of 5’1.1 kb, 3’6.1 kb and exon 16.

A) illustrates overexpression of 5’1.1 kb, then exon 16 (B) and 3’6.1 kb (C) were downregulated with reference to the other two genes (exon 16 and 5’1.1 kb).
Figure 3.45: Melting curve analysis of 5’1.1 kb, 3’6.1 kb and exon 16.

Specificity of LightCycler PCR for amplification of 5’1.1 kb (A), Exon 16 (B) and 3’6.1 kb (C) as determined by melting curve analysis. Melting peaks were determined by plotting the continuous negative derivative of fluorescence emitted by each sample as PCR products were slowly melted. The no-template control sample (D) showed some fluorescence confirming the presence of primer dimers.