# A morphologic and immunohistochemical analysis of gastric adenocarcinoma with regard to the presence of E-cadherin and localisation of β-catenin staining

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# Table of contents

# Declaration

I, Dr Riyaadh Roberts, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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# Abstract

**Introduction:** Surgical resections for gastric carcinoma are a commonly encountered histopathologic specimen. Despite this, gastric carcinoma in South Africa and particularly within the Western Cape Province has not been well documented.

**Aims:** To interrogate the WNT signalling pathway using selected protein expression by immunohistochemistry and correlating these with tumour subtypes.

To assess Her2/neu expression and correlate this with morphologic subtypes

**Objectives:** To determine aberrations in the expression of β-catenin, E-cadherin (extracellular and cytoplasmic domains) and DVL1 in gastric carcinoma subtypes. Additionally, to determine Her2/neu overexpression by immunohistochemistry and correlate this with morphologic subtype.

**Materials and methods:** 97 gastric adenocarcinoma resection cases were retrieved and stained with antibodies against β-catenin, DVL1, E-cadherin (extracellular domain), E-cadherin (cytoplasmic domain) and Her2/neu. Results were considered statistically significant if $P < 0.05$.

**Results:** All 97 cases were confirmed as gastric adenocarcinomas, with 39 (40%) intestinal type, 51 (53%) diffuse type and 7 (7%) mixed type tumours identified respectively. Patient ages ranged from 18 to 84 years. Her2/neu was overexpressed in 12 (12%) cases, with 9 of these cases showing intestinal-type morphology ($P = 0.0174$). Abnormal β-catenin localisation occurred in 14 (14%) cases. Aberrant E-cadherin (extracellular domain) localisation or absent staining was seen in 36 (37%) cases, with a significant proportion demonstrating diffuse type morphology ($P <0.001$). Abnormal E-cadherin (cytoplasmic domain) localisation or absent staining was seen in 7 (7%) cases, with a significant 6 of these 7 cases showing diffuse-type morphology ($P = 0.0231$). Eleven (11%) cases were seen in patients younger than 40 years, with 9 of these cases showing intestinal morphology and a significant 7 of the 11 cases showing aberrant E-cadherin (extracellular domain) localisation ($P = 0.025$).

**Conclusion:** Gastric adenocarcinomas show derangements in the WNT signalling pathway. Distinct immuno-morphologic correlations are apparent in tumours demonstrating Her2/neu overexpression or abnormal E-cadherin localisation. Tumours occurring in younger individuals show intestinal morphology, poor differentiation and E-cadherin abnormalities.

# List of abbreviations

APC – α-fetoprotein

APES - aminopropyltriethoxysilane

aPKC - atypical protein kinase C

Cag A - Cytotoxin associated gene A

CANSA - Cancer Association of South Africa

CEN17 – Centromere on chromosome 17

CK1α - Casein kinase 1 α

CpG – Cytosine phosphate guanine

DNA - Deoxyribonucleic acid

DVL - Dishevelled

EBV – Epstein Barr virus

FAP - Familial adenomatous polyposis

FZD - Frizzled

GSK3β - Glycogen synthase kinase 3 beta

H&E – Haematoxylin and eosin

*H.pylori – Helicobacter pylori*

Her2/neu - Human epidermal growth factor receptor gene 2

HNPCC - Hereditary non-polyposis colorectal carcinoma

IL-1β - interleukin 1 beta

*Int1* - Murine mammary tumour oncogenic integration site gene

LRP - Low density lipoprotein receptor related protein

MMed – Master of Medicine

NHLS - National Health Laboratory Service

NRF - National Research Fund

NFAT - Nuclear factor associated with T cells

Obj mag – Objective magnification

PBS – Phosphate buffered saline

TCF/LEF - T cell factor/lymphoid enhancer factor

TNF-$\alpha$ - Tumour necrosis factor alpha

SEER – Surveillance, Epidemiology and End Results

SOX9 - Sex determining region Y box 9

SRFP - Secreted frizzled-related proteins

UCT - University of Cape Town

Vac A - Vacuolating gene A

*Wg - wingless*

# 1 Introduction and Literature review

## 1.1 Introduction

Gastric carcinoma is a malignant epithelial neoplasm that is not uncommonly encountered in histopathology resection specimens, with adenocarcinoma representing 80-90% of all types of gastric carcinoma. [1] These neoplasms demonstrate distinct differences in their immunohistochemical profile and prognosis, dependent in part, on their morphology, demographic distribution and underlying genetic aberrations.

The occurrence of gastric carcinoma in South Africa and in particular within the Western Cape Province has not been well documented. The following literature review takes into account the current worldwide knowledge base regarding the specific demographics, aetiopathogenesis, morphology, immunohistochemical staining profile and molecular pathways of gastric adenocarcinoma and compares these features to cases found within the Western Cape.

## 1.2 Historical perspective

During the late 1970's and early 1980's, gastric adenocarcinoma was considered the commonest primary malignant epithelial neoplasm, with up to 650 000 new cases reported per year. [2] In 2002, this figure increased to 930 000 new cases of gastric carcinoma per year. [3] This is however no longer the case in the current socioeconomic climate, with lung cancer now the leading malignant cancer worldwide. There has been a steady decline in the frequency of gastric adenocarcinoma, in part due to the understanding of its association (and subsequent antibiotic and medical treatment) with *Helicobacter pylori* (*H.pylori*) and the role of refrigeration (with a decline in salt-based food preservation). [4]

# 1.3 Epidemiology

## 1.3.1 Incidence and geographic distribution

Gastric carcinoma remains a leading cause of death worldwide, being the fourth most common cause of cancer related death. [5] The incidence varies widely, with defined high risk and low risk areas having been identified. High risk areas include Japan, China, Chile and Portugal. Low risk areas include the United States of America and the United Kingdom. [6] South Africa is considered a low-to-intermediate risk region. [3] In Asian countries, Japan has the highest incidence rates of gastric carcinoma, with up to 115 cancers per 100 000 population per year. Portugal has the highest incidence amongst Western European countries, with 33 cancers / 100 000 population per year. Comparatively, South Africa has an incidence rate of 11.9 cancers per 100 000 population per year. [7] The Lauren classification divides gastric adenocarcinoma into intestinal and diffuse subtypes, based on morphology. The intestinal subtype appears to predominate in high risk areas and arises from precursor lesions. However the diffuse type of gastric adenocarcinoma does not exhibit a geographic predominance and no definitive precursor lesion has been identified. In addition, patients in low risk areas develop proximal (cardia and fundal) tumours, while distal (antropyloric) tumours predominate in high risk regions. [103]

## 1.3.2 Age

Gastric carcinoma is predominantly a disease of middle aged to elderly individuals. Most patients present between the ages of 40 and 70 years, with a mean age at presentation of 52 years. Gastric carcinoma in patients younger than 40 years (early-onset gastric carcinoma) is uncommon, accounting for less than 5% of cases. [8, 9] These early-onset cases differ in their sex incidence (with either an equal male-to-female ratio or female predominance), morphology, (diffuse type rather than intestinal type), poor differentiation and generally have a poor prognosis. [8, 9, 105] Matley et al, 1988 [8] showed that this also holds true

for cases seen within the Western Cape in South Africa. Additionally, some cases of early onset gastric carcinoma have been shown to harbour a germline mutation in the *CDH1* gene, encoding for E-cadherin. [10]

### 1.3.3 Gender

Gastric carcinoma shows a strong male predominance, with an approximately 2:1 male to female ratio. [102] The male prevalence of gastric carcinoma is greater in high incidence areas. Powell and McConkey, 1990 [11] showed a consistently higher male-to-female ratio in gastric carcinomas arising in the cardia compared to those arising in the distal stomach (antrum and pylorus). Gastric carcinoma occurring in patients younger than 40 years, show a 1:1 or 0.9:1 male-to-female ratio. [8, 9]

### 1.3.4 Race and socio-economic status

Worldwide, gastric adenocarcinomas are more common in Asians and Blacks than in Caucasians. [12] Within South Africa, many more Coloured individuals are affected by the disease, with 98 cancers / 100 000 population. [13] Additionally, worldwide and within Southern Africa, increased rates of gastric adenocarcinoma are noted in lower socioeconomic groups. [14]

# 1.4 Aetiopathogenesis

Gastric carcinoma is a multifactorial and multistep process that often involves a step-wise progression from normal gastric mucosa, though chronic gastritis, atrophic gastritis and intestinal metaplasia, dysplasia, carcinoma in-situ and ultimately to invasive carcinoma.

Risk factors commonly implicated in the development of the disease can be subdivided into three broad categories.

- **Environmental factors**

The most important factor in the development of gastric carcinoma is infection by *H.pylori*. Diets rich in salts and nitrites and diets low in antioxidants have also been implicated in carcinogenesis through the formation of carcinogenic N-nitroso compounds. Rare cases of Epstein-Barr virus (EBV) associated gastric carcinomas have been reported. [15]

- **Host factors**

The commonest host factors include chronic atrophic gastritis with intestinal metaplasia, partial gastrectomy with bile reflux and the presence of gastric adenomas with high grade dysplasia. Autoimmune gastritis and Menetrier's disease are considered uncommon precursor lesions in gastric adenocarcinoma development. [16]

- **Genetic factors**

Germline mutations in the *CDH1* gene (encoding E-cadherin), [10] a family history of gastric adenocarcinoma, the presence of Hereditary Non-polyposis Cancer Syndrome (HNPCC) [17] and Familial Adenomatous Polyposis (FAP) [18] are risk factors strongly associated with the development of this malignancy.

### 1.4.1 *H.pylori* in gastric carcinoma

*H.pylori* plays an integral role in the development of gastric adenocarcinomas through a complex interaction between bacterial virulence factors, cytokines, free radicals and host immunity. [19] *H.pylori* is a Gram-negative curvilinear coccobacillus that resides within the gastric mucus of infected individuals. It produces a number of factors that disrupt the normal mucosal barriers of the stomach and act as promoters of carcinogenesis

These include:

- **Production of urease**

Urease is a bacterial produced enzyme that results in the metabolism of urea to ammonia and carbon dioxide, thereby buffering gastric acid in the immediate vicinity of the organism. Ammonia gets further degraded to carcinogenic nitrate and nitrite intermediates via the nitrogen cycle

- **Cytotoxin associated gene A (Cag A )**

Cag A is a virulence factor associated with increased colonisation of mucus by organisms, increased epithelial damage, increased inflammatory response and increased carcinogenic potential. Cag A-positive strains of *H.pylori* are associated with an increased incidence of distal gastric carcinoma while being rare in gastric cardia carcinoma. [20]

- **Vacuolating gene A (Vac A)**

Vac A is an associated virulence factor which requires Cag A for normal functioning. The combination of Cag A and Vac A results in an intracytoplasmic passive transport system, with intracytoplasmic vacuole formation and increased urea transport to the surface epithelium. The end result is an increased urea load, with subsequent urease degradation within the gastric mucus and ultimately increased levels of ammonia.

- **Phospholipase production**

Phospholipase is a bacterial produced enzyme that causes destruction of the cytoplasmic membrane phospholipid bilayer of gastric epithelial cells. This decreases intrinsic host defences and causes seepage of host intracellular metabolites into the mucus, providing nutrients to the bacilli.

- **Protease production**

Similar to phospholipase, protease is a bacterial produced enzyme that causes destruction of glycolipid complexes within the gastric mucus. This causes alteration of normal gastric mucus structure, thus decreasing intrinsic host defences and rendering the gastric epithelial cells more prone to bacterial damage.

- **Upregulation of host immunity and hypochlorhidria**

*H.pylori* infection results in the production of the pro-inflammatory cytokines interleukin 1, interleukin 6, interleukin 8 and tumour necrosis factor alpha (TNF-α) by mucosal epithelial cells, with subsequent recruitment and activation of neutrophils. Enhanced expression of interleukin 1 beta (IL-1β) is an inhibitor of gastric acid secretion, resulting in decreased gastric acid production and hypochlorhidria. Increased colonisation of gastric mucus by *H.pylori* organisms results in a pangastritis, development of atrophic gastritis and subsequent increased risk of gastric carcinoma. [21] Additionally, IL-1β further upregulates TNF-α, which is also an inhibitor of gastric acid production. [22]

- **Increased free radical production**

The active inflammation associated with *H.pylori* infection results in the production of free radicals and activation of nitric oxide. [23] Nitric oxide further combines with these oxygen-derived free radicals to form highly reactive oxygen species that directly damage gastric epithelial cells and cause mutations within DNA. [24] Accumulated mutations provide a fertile medium for neoplastic transformation.

- **Decreased gastric anti-oxidant levels**

A diet rich in fresh fruits and vegetables, combined with increased intake of vitamin C and vitamin E, aid in scavenging oxygen-derived free radicals, thus preventing the formation of carcinogenic N-nitroso compounds. Studies have shown that, by mechanisms which are unclear, *H.pylori* infection results in decreased levels of vitamins C and E both within gastric epithelial cells and within the gastric mucus. [25, 26]

- **Increased epithelial cell proliferation and increased risk of mutations**

*H.pylori* associated chronic gastritis represents an epithelial hyperproliferative state, with expansion of the proliferative zone within gastric pits. [27] The normally non-dividing Paneth cells and goblet cells (which occur in the setting of intestinal metaplasia) are also noted to show increased proliferative activity, implying a component of cell-cycle dysregulation and increased propensity to carcinogenic inducing mutations. [28]

In addition to the above factors, *H.pylori* plays an integral role in activation of the WNT signalling pathway through activation of β-catenin. [29]

## 1.4.2 The WNT signalling pathway

The WNT's represent a ubiquitous family of protein ligands that play a critical role in embryogenesis, cell migration, tissue homeostasis and neoplasia. The term, WNT, was first coined by Nusse et al [30] in 1991 to describe a combination of the homologous *wingless (wg)* gene discovered on the *Drosphilia menalogaster* fruitfly and the *Int1 (Wnt1)* murine mammary tumour oncogenic integration site gene. [31] Recessive mutations in *wg* are associated with failure of wing development in fruitflies, while *Int1* aberrations cause mammary carcinoma in mice.

### 1.4.2.1 Members of the WNT family

There are currently 19 human *WNT* genes, as defined by their amino acid sequences. [32] These *WNT* genes are: *WNT1, WNT2, WNT2B, WNT3, WNT3A, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT9A, WNT9B, WNT10A, WNT10B, WNT11 and WNT16.* [33]

WNT's are glycoproteins with characteristic spacing of cysteine amino acid residues [34] that bind to receptors with cysteine-rich domains. These receptors include, amongst others, Frizzled (FZD), low density lipoprotein receptor related protein (LRP) and transmembrane tyrosine kinases.

FZD receptors are G-protein coupled receptors. Ten FZD receptors are currently identified in humans. The N-terminal of FZD receptors represents the cysteine-rich WNT binding domain. Secreted frizzled-related proteins (SRFP) are the secreted forms of FZD that bind WNT. Upon binding to WNT, they act as inhibitors of the pathway.

LRP5/6 is a member of the low density lipoprotein receptor related protein (LRP) family and plays an integral role in WNT signalling through co-binding of WNT ligands to the members of the FZD family.

### 1.4.3 The canonical WNT signalling pathway

Two closely linked and inter-related pathways exist in WNT signalling. The first pathway is a β-catenin dependent pathway, known as the canonical pathway.

Two β-catenin pools are present within human cells. The membrane-bound pool, through its interaction with the E-cadherin cell adhesion molecule, aids in cell-cell interaction by binding to the actin cytoskeleton. A second, WNT regulated β-catenin pool is present within the cytoplasm.

In the absence of WNT signalling, β-catenin is phosphorylated, resulting in its eventual proteosomal degradation via an E3 ubiquitin ligase process. [35] Glycogen synthase kinase 3β (GSK3β) and Casein kinase 1 α (CK1α) mediate the process of phosphorylation by interaction with Axin and Adenomatous polyposis coli (APC) proteins, ultimately forming an APC-Axin- GSK3β-CK1α complex

With WNT binding to FZD, via the LRP5/6 co-receptor, there is formation of a WNT-FZD-LRP5/6 trimer. Subsequently, FZD binds to Dishevelled (DVL) and there is phosphorylation of the cytosolic tail of LRP5/6. The phosphorylated LRP5/6 then binds to Axin and inactivates the APC-Axin-GSK3β-CK1α complex. β-catenin remains unphosphorylated and accumulates within the cellular cytoplasm. Nuclear translocation of β-catenin then occurs, whereupon it acts as a potent transcription co-regulator through its interaction with nuclear transcription factors. β-catenin associates with T cell factor/lymphoid enhancer factor (TCF/LEF) family transcription factors (TCF1, LEF1, TCF3 and TCF4) that subsequently activate downstream target genes. These target genes include, c-myc and cyclin D1, resulting in cellular proliferation. [Figure 1] [36]

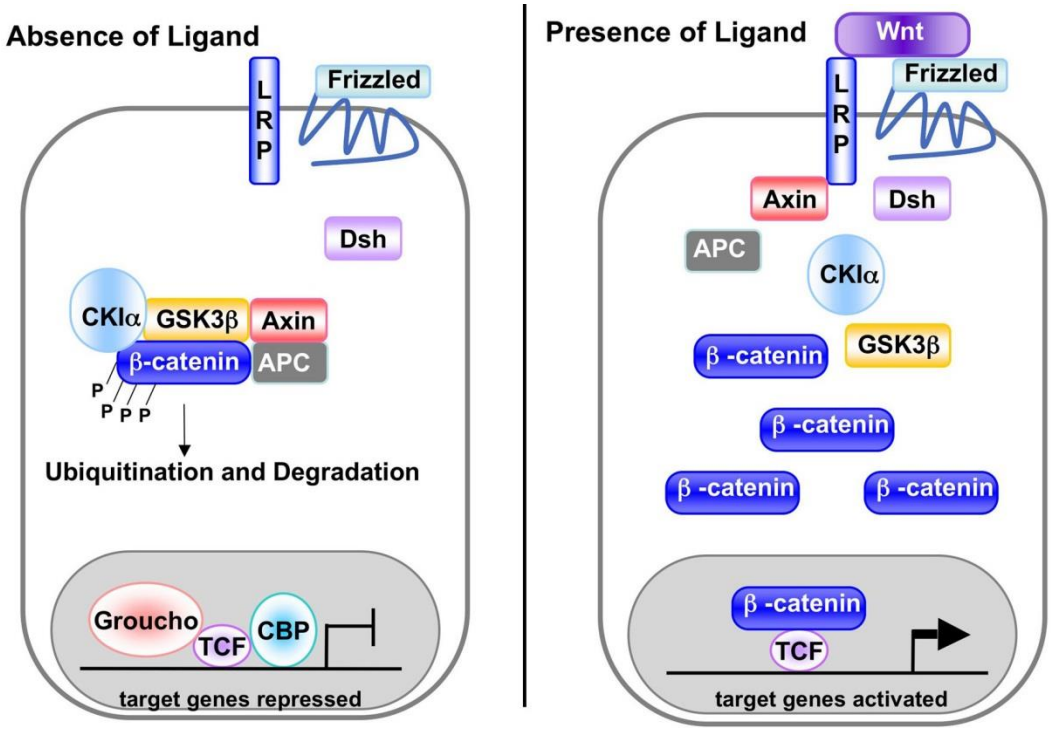



Figure 1: Schematic representation of the WNT signalling pathway

In an attempt to prevent inappropriate WNT signalling, both intracellular and extracellular regulators and inhibitors of WNT signalling are present. Intracellular regulators include Sex determining region Y box 9 (SOX9). Extracellular inhibitors are multiple and include, amongst others, SRFP [37] and the Dickkopf [33] family of genes.

The canonical WNT pathway plays a critical role in stem cell maintenance and tissue development in both foetal and adult tissues. [38] Aberrations in this canonical pathway by abnormal constitutive activation (through ligand over-expression or down-regulation of intracellular or extracellular inhibitors), results in a variety of malignant neoplasms. [36]

### 1.4.4 The non-canonical WNT signalling pathways

The non-canonical WNT signalling pathways regulate critical events during embryogenesis. The mechanisms that underlie the activation of the non-canonical pathway are poorly understood.

The non-canonical pathways are multiple and are best defined as pathways that are WNT and FZD mediated, but independent of β-catenin transcriptional activity. [39] The three components that comprise this pathway are the: WNT-polarity pathway, WNT-$Ca^{2+}$ pathway and WNT-atypical protein kinase C pathway, mediated by WNT family members *WNT4, WNT5A* and *WNT11*. These pathways are involved in the development of planar cell polarity in *Drosphilia,* and neuronal and epithelial cell migration.

The WNT polarity pathway ensures normal epithelial and neuronal cellular polarity, mediated by interactions with DVL and FZD. [40, 41]

The WNT-$Ca^{2+}$ pathway is mediated by WNT binding to FZD, resulting in G-protein coupled receptor activation. Intracellular calcium is released with subsequent activation of $Ca^{2+}$ dependent transcription factor nuclear factor associated with T cells (NFAT). [42] Additionally, there is paradoxical interaction with the canonical pathway, whereby the WNT-$Ca^{2+}$ pathway serves as an inhibitor of β-catenin, preventing canonical signalling.

The WNT-atypical protein kinase C pathway is a poorly understood regulatory mechanism of neuronal/neuroectodermal differentiation and ordered assembly. It involves a complex series of interactions between DVL and atypical protein kinase C (aPKC), whereby DVL directly regulates aPKC activity in hippocampal neurons. Downregulation of DVL reduces axon differentiation while DVL overexpression induces formation of multiple axons. [43]

### 1.4.5 WNT signalling in human diseases and cancer

WNT's play an integral role in cellular movement, cellular polarity, cellular proliferation and cellular destruction in both adult and stem cells. [30] Mutations in *WNT* genes are associated with developmental abnormalities, while aberrations in WNT signalling pathways are associated with neoplasia and certain multisystemic diseases. (Table 1)

Table 1: WNT genes associated with human disease (modified from Logan and Nusse, 2004) [44]

| Gene | Human disease |
|---|---|
| *WNT5a* | Gastric carcinoma [79] |
| *APC* | Gastric carcinoma [57], Polyposis coli [51], colon cancer [52] |
| *WNT3* | Tetra-amelia [45] |
| *LRP5* | Bone density defects [46], Vascular defects in the eye (osteoporosis-pseudoglioma syndrome; OPPG) [47], familial exudative vitreoretinopathy; (FEVR) [48] |
| *FZD4* | FEVR [48, 49] |
| *Axin2* | Tooth agenesis,<br>Predisposition to colorectal cancer [50] |

Genetic alterations, constitutive activation and mutations of genes within both the canonical and non-canonical WNT signalling pathways are associated with human cancer development [Table 2]. [53] Colorectal adenocarcinoma is a well-studied and frequently encountered histopathologic specimen, demonstrating mutually exclusive mutations in *APC* and *β-catenin*. *APC* mutations are encountered in colorectal adenocarcinomas occurring in the setting of familial adenomatous polyposis (FAP), as well as approximately 80% of sporadic colorectal adenocarcinomas. [54] Up to 10% of sporadic colorectal adenocarcinomas demonstrate a mutation in β-catenin. [55] Additionally, *β-catenin* mutations are noted in approximately 40% of HNPCC-associated colorectal adenocarcinomas. [56]

*β-catenin* mutations are frequently associated with invasive and non-invasive human neoplasms. These include pancreatic solid pseudopapillary tumours, [65] hepatocellular carcinomas, medulloblastomas [54], ovarian endometrioid borderline tumours and ovarian invasive endometrioid adenocarcinomas [66] and pulmonary blastoma. [67]

In gastric adenocarcinoma, *β-catenin* mutations (particularly mutations within exon 3 of the *β-catenin* gene) occur in 25 - 60 % of cases. [98, 99] These mutation positive cases can be detected immunohistochemically by the presence of aberrant nuclear β-catenin staining. [68] A genotype-phenotype correlation is present, with a strong association noted between nuclear β-catenin accumulation and intestinal type morphology. [101]

Linked to β-catenin in the canonical WNT pathway is E-cadherin. Downregulation of E-cadherin and loss of immunohistochemical membrane staining is seen in diffuse-type gastric adenocarcinomas [70] and lobular breast carcinomas. [71]

Table 2: Genetic alterations in WNT signalling (modified from Polakis, 2012) [53]

| Affected gene | DNA/mRNA Alteration | Functional outcome | Cancer type |
|---|---|---|---|
| *CTNNBl* (β-catenin) | Missense/in-frame Deletion | Enhanced protein Stability | Gastric [68, 98, 99] Hepatocellular/ Medulloblastoma [54] |
| *APC* (APC) | Truncation | Reduced regulatory Activity | Gastric/Colorectal[57] Gastric [100, 101] |
| Axins (*Axin I, Axin II*) | Truncation/ Missense | Reduced regulatory Activity | Hepatocellular/ Colorectal [58] |
| CREBP(CBP) | Truncation/ Missense | Inactive Acetyltransferase | Lymphoma/ Leukaemia [59] |
| GSK3b | Mis-splicing, inframe Deletion | Inactive kinase | Leukaemia [60] |
| LRP5 | Mis-splicing, inframe Deletion | Loss of repression by DKK1 | Breast/parathyroid [61] |
| *TCF7L2* (TCF4) | Missense/deletion/ Truncation | Loss of repression | Colorectal [62] |
| *TCF7L2* (TCF4) | Fusion with VT11A Gene | Unclear | Colorectal [63] |
| FAB123B (WTX) | Truncation/ Deletion | Loss of function | Wilms tumour [64] |

Both the canonical and non-canonical WNT signalling pathways play a critical role in gastric adenocarcinoma. Studies have shown that β-catenin independent non-canonical signalling with increased expression of *WNT5A* is associated with aggressive behaviour in gastric adenocarcinoma, by stimulation of cell migration and enhancing the invasive capabilities of the tumour cells. [79]

Additionally, *β-catenin* mutation (canonical pathway) is a frequent cause of inappropriate activation of the WNT pathway in gastric carcinoma. [68] In an attempt to prevent this inappropriate WNT signalling, both intracellular and extracellular inhibitors of WNT signalling are present. Secreted frizzled-related protein 1 (SRFP) serves as a critical extracellular inhibitor. Studies have linked epigenetic inactivation of SFRP genes to constitutive activation of WNT signalling in gastric cancer. [81]

### 1.4.6 The role of E-cadherin in gastric carcinoma

Epithelial-cadherin (E-cadherin) is a member of the cadherin superfamily, serving as the main epithelial intercellular adhesion molecule. It is encoded by *CDH1,* a gene present on the long arm of chromosome 16. The encoded protein is a calcium dependent cell-cell adhesion molecule. E-cadherin plays a critical role in the normal architecture of epithelial tissues and in cell differentiation through its interaction with the catenin group of molecules. [95] E-cadherin consists of 5 repeats within the extracellular domain, a transmembrane domain and a phosphorylated intracellular domain. This intracellular domain links E-cadherin to the WNT signalling pathway via its binding of p120 and β-catenin.

Closely aligned to tumour stromal invasion is the process of epithelial-mesenchymal transition. In a normal state, E-cadherin acts as a tumour suppressor gene through maintenance of cell-cell adhesion and sequestration of β-catenin on the cell membrane. When E-cadherin expression is downregulated, the loss of E-cadherin results in release of β-catenin into the cytoplasm [94]. Subsequent translocation of β-catenin into the nucleus occurs and ultimately results in the upregulation of epithelial-mesenchymal related transcription factors. These transcription factors cause a change in phenotype from a cohesive epithelial cell to a motile and discohesive mesenchymal cell that has increased stromal invasive abilities.

Downregulation of E-cadherin can occur as a result of *CDH1* gene mutations (both germline and somatic), CpG island promoter hypermethylation or epigenetic silencing (through the actions of *Snail*, *Slug* and *Twist*). [83]

This downregulation of E-cadherin expression occurs in the malignant progression of carcinomas and is seen in lobular breast carcinomas and approximately 50% of diffuse type gastric adenocarcinomas. [81]

Hereditary diffuse gastric carcinoma is an autosomal dominant condition caused by a germline mutation in *CDH1*. It accounts for less than 1% of all gastric malignancies and is characterised by gastric carcinoma occurring in patients younger than 40 years, loss of E-cadherin expression, diffuse type morphology and early death. [104]

Loss of E-cadherin expression (which can be demonstrated by immunohistochemical staining) is associated with poor tumour differentiation, increased tumour grade, increased metastasis and poorer prognosis. [81, 82, 83, 90]

### 1.4.7 Dishevelled in gastric carcinoma

Dishevelled (DVL) is a cytoplasmic phosphoprotein that acts downstream of FZD receptors in the WNT signalling pathway. Three human DVL genes have been identified, namely *DVL1*, *DVL2* and *DVL3*. [96]

*DVL1* is a candidate gene in the process of neuroblastomatous development and aberations in *DVL1* may play a role in the development and phenotypic manifestations of Charcot-Marie-Tooth disease.

Though the role of DVL in the WNT signalling pathway is well established, [96, 97] its direct role in gastric carcinoma pathogenesis is not well elucidated.

### 1.4.8 Human epidermal growth factor receptor gene 2 (Her2/neu) and targeted therapy in gastric carcinoma

Her2/neu is an oncogene on chromosome 17q12–q21, encoding a 185-kDa transmembrane tyrosine kinase receptor (p185), which is a member of the epidermal growth factor receptor family.

Approximately 20% of gastric adenocarcinomas overexpress Her2/neu. Overexpression of Her2/neu can be assessed by both immunohistochemistry and fluorescent in-situ hybridisation (FISH) techniques, with a strong concordance between the two. [87]

Molecular therapy targeting Her2/neu (anti Her2/neu monoclonal antibody Trastuzumab/Herceptin) is currently approved for the treatment of advanced gastric adenocarcinoma and metastatic gastric adenocarcinoma, if the carcinoma demonstrates unequivocal evidence of Her2/neu overexpression (by immunohistochemistry) or amplification (by FISH).

The multinational ToGA trial validated the Her2/neu testing criteria for the determination of overexpression and amplification, [84] with immunohistochemically determined overexpression equating to a score of 3+ and FISH determined amplification by a Her2:CEN17 ratio of greater than two (Her2 : CEN17 >2.0).

Large studies have evaluated the significance of Her2/neu expression in gastric carcinoma and its effect on patient survival, with conflicting data emerging. Marx et al, 2009 [85] and Kunz et al, 2011 [86] showed no association between these parameters. However, studies by Kim et al, 2007 [87], Yan et al, 2010 [88] and Choong Kim et al, 2011 [89] showed that Her2/neu overexpression was associated with decreased patient survival and an overall poorer prognosis.

No correlation between Her2/neu overexpression/amplification and tumour stage has been established. However, a strong morphologic correlation with Her2/neu overexpression/amplification is present. Using this parameter, intestinal type tumours showed a higher rate of Her2/neu positivity (32%) than diffuse type carcinomas (6%). [85-87]

# 1.5 Clinical features

## 1.5.1 Presenting features

Gastric adenocarcinoma is a disease of insidious onset with variable clinical features and a frequent initial asymptomatic period. The initial presentation is often non-specific with vague upper gastrointestinal symptoms including anorexia, nausea, vomiting and dyspepsia. Patients with more advanced lesions complain of weight loss, an epigastric mass, dysphagia, haematemesis and melaena. A subcutaneous umbilical nodule (Sister Mary Joseph nodule) may be present and represents a periumbilical metastatic deposit. Additionally, there may be supraclavicular lymphadenopathy (Virchow Trossier node) due to lymph node metastasis. Distinctive bilateral ovarian gastric adenocarcinoma metastases can occur (Krukenburg tumour).

## 1.5.2 Diagnosis

Gastric carcinoma is diagnosed on gastroscopy with biopsy, by biopsy of a peripheral metastatic lesion or by biopsy or resection of a suspicious intra-abdominal lesion at the time of laparotomy.

## 1.5.3 Surgical resection

Surgical resection offers the possibility of a cure for gastric carcinoma. For tumours limited to the antrum and-/-or pylorus, a partial gastrectomy is the preferred surgical procedure. This may be of Billroth I (pyloric resection with anastomosis of gastric body to duodenum) or Billroth II (gastric antrectomy with gastrojejunostomy) type. Total gastrectomy is performed for proximal tumours, while oesophagogastrectomy is performed for tumours arising at the gastro-oesophageal junction. All these surgical resections include a regional lymphadenectomy.

# 1.6 Pathology

## 1.6.1 Macroscopic pathology

On gross pathology, gastric carcinomas may be nodular, fungating, ulcerating or polypoid. A distinct infiltrative subtype (linitis plastica) results in widespread fibrotic thickening of the gastric wall, resembling a thickened leather bottle.

Ulcerated carcinomas occur most frequently within the pyloric antrum and lesser curve. These ulcers have ragged, raised and rolled edges.

Polypoid, nodular and fungating tumours consist of friable masses, projecting into the gastric lumen. These tumours tend to occur along the greater curve and fundus.

## 1.6.2 Microscopic pathology

The vast majority of tumours are adenocarcinomas, composed of either nests, tubules, trabeculae, papillae or discohesive epithelial cells with intracytoplasmic mucin vacuoles. Cellular pleomorphism is variable, depending on the subtype and degree of differentiation. For all types of advanced adenocarcinoma (excluding early gastric carcinoma), there is invasion into and beyond the submucosa. Invasive malignant cells may extend to the muscularis propria, serosa or into adjacent organs.

## 1.6.3 Classification systems

Numerous gastric carcinoma classification systems exist. These include: The World Health Organisation (WHO) system [72], Lauren classification [73], Ming classification [74], Mulligan and Rember classification [75], Goseki classification [76] and Carnerio classification. [77]

The WHO and Lauren classification systems are the most widely used.

### 1.6.3.1 The WHO classification system

The typing of gastric adenocarcinoma is based on the predominant histomorphology of the tumour. [72] The subtypes include:

- Papillary adenocarcinoma
- Tubular adenocarcinoma
- Mucinous adenocarcinoma
- Signet-ring adenocarcinoma

Papillary adenocarcinomas are composed of finger-like outgrowths of neoplastic glandular epithelium with variable pleomorphism, overlying fibrovascular cores.

Tubular adenocarcinomas consist of branching glands with tubular architecture, lined by atypical neoplastic glandular epithelium and embedded within a fibrous stroma.

Mucinous adenocarcinomas are adenocarcinomas with abundant pools of extracellular mucin, comprising more than 50% of the tumour. Well differentiated tumours form small glands lined by columnar epithelium. Poorly differentiated tumours show linear cords and discohesive epithelial cells with intracytoplasmic vacuoles, present within pools of mucin.

Signet-ring adenocarcinomas are composed of discohesive epithelial cells with a large intracytoplasmic mucin vacuole, causing eccentric displacement of the nucleus. Additionally, atypical cells with granular eosinophilic cytoplasm and neutral mucin, as well as epithelial cells with absent intracytoplasmic mucin, are seen. No tubule formation occurs.

### 1.6.3.2 The Lauren classification system

Two subtypes of gastric adenocarcinoma [73] are present:

- Intestinal-type
- Diffuse-type

The intestinal-type is composed of large pleomorphic, mitotically active epithelial cells with large nuclei, prominent nucleoli and variable amounts of intracytoplasmic mucin. The tumour cells form glands, nests, sheets, tubules and may demonstrate papillary architecture.

The diffuse-type is predominantly composed of poorly cohesive or discohesive epithelial cells with mild nuclear hyperchromasia and minimal pale eosinophilic-to-clear cytoplasm, often infiltrating into a desmoplastic stroma. Signet-ring morphology is often apparent. Gland formation is inconspicuous, but may be appreciated within the superficial regions of the tumour.

### 1.6.4 Gastric carcinoma staging

Gastric carcinomas are staged according to the 2009 7th edition TNM tumour staging system [78], which characterises the extent of the lesion according to the parameters of size of the primary tumour (T), regional lymph node involvement (N) and distant metastatic spread (M).

T – primary tumour

pT1 – Tumour invades lamina propria or submucosa

pT2 – Tumour invades muscularis propria or subserosa

pT2a - tumour invades muscularis propria

pT2b - tumour invades subserosa

pT3 – tumour penetrates the serosa (visceral peritoneum) without invasion of adjacent structures.

PT4 – tumour invades adjacent structures

N – regional lymph nodes

pN0 – No regional lymph node metastasis

pN1 – Metastasis in 1-6 regional lymph nodes

pN2 - Metastasis in 7-15 regional lymph nodes

pN3 - Metastasis in more than 15 regional lymph nodes

M – Distant metastasis

M0 – No distant metastasis

M1 - Distant metastasis present

# 1.7 Early gastric carcinoma

Early gastric carcinoma is defined as a carcinoma limited to the mucosa (mucosal subtype); or the mucosa and submucosa only (submucosal subtype), irrespective of the presence of lymph node metastases. When compared to advanced gastric carcinomas, these tumours have a much better prognosis, with up to 90% 5 year survival. [91]

## 1.7.1 Macroscopic pathology and classification

Early gastric carcinoma is classified according to the Japanese Gastroenterological Endoscopic Society guidelines, which are based on the macroscopic endoscopic appearance of the tumour. (Table 3).

Table 3. Macroscopic classification of early gastric carcinoma

| | |
|---|---|
| Type I | Protruded type<br>- A polypoid, nodular or villous tumour that projects into the lumen |
| Type II | Superficial type |
| - Type IIa | Elevated subtype<br>- A well circumscribed plaque-like lesion that is slightly elevated above the mucosal surface |
| - Type IIb | Flat subtype<br>- No macroscopic abnormality visible |
| - Type IIc | Depressed subtype<br>- The surface of the lesion is slightly depressed below the adjacent mucosa |
| Type III | Excavated type<br>- Ulceration into the underlying gastric wall |

# 2.Study design

The occurrence of gastric carcinoma in South Africa and in particular within the Western Cape Province has not been well documented. This study took into account the current worldwide knowledge base regarding the specific demographics, morphology and immunohistochemical staining profile of gastric adenocarcinoma and compared these features to cases found within the Western Cape.

## 2.1 Study aims

The aims of this study are as follows:

- To interrogate the WNT signaling pathway using selected protein expression by immunohistochemistry and correlating these with tumour subtypes.

- To assess Her2/neu expression and correlate this with morphologic subtypes

## 2.2 Study objectives

To determine aberrations in the expression of β-catenin, E-cadherin (extracellular and cytoplasmic domains) and DVL1 in gastric carcinoma subtypes. Additionally, to determine Her2/neu overexpression by immunohistochemistry and correlate this with morphologic subtype.

## 2.3 Ethics approval

Ethics approval was obtained from the University of Cape Town (UCT) Faculty of Health Sciences Human Research Ethics Committee (Reference number 504/2009) as part of the broader study proposal titled "Molecular analysis and identification of biomarkers from formalin fixed paraffin embedded gastric cancers". The research proposal for this MMed project obtained scientific approval from both the Department of Clinical Laboratory Sciences Research Committee and the Faculty of Health Sciences Research Committee at UCT.

Funding for this study was obtained from the Cancer Association of South Africa (CANSA), National Health Laboratory Service (NHLS) and National Research Fund (NRF).

## 2.4 Materials and methods

### 2.4.1 Acquisition of cases

A retrospective study was undertaken. A computerised DISA search of the database of the Division of Anatomical Pathology, University of Cape Town / National Health Laboratory Service was performed, searching for all cases of gastric carcinoma resections between January 2003 and December 2011.

97 gastric adenocarcinoma resection cases were identified. Cases were allocated study numbers and patients' names and other identification details were anonymised. Archived stained slides of the cases were retrieved and reviewed. The diagnosis in each case was confirmed and the morphological data recorded. Archival tissue blocks were retrieved and additional sections cut for immunohistochemistry.

The pathology reports of the cases studied were consulted to document the patient's age and gender; type of procedure; size and mass of the gross specimen; and the morphologic subtype of tumour.

## 2.4.2 Antibodies

The following primary antibodies were used (Table 4): β-catenin, DVL1, E-cadherin extracellular domain (36B5), E-cadherin cytoplasmic domain (36/E-cadherin) and Her2/neu.

Table 4: Primary antibodies

| **Primary antibody** | **Clone** | **Supplier** | **Antigen retrieval** | **Dilution** | **Incubation time (min)** | **Positive external control** |
|---|---|---|---|---|---|---|
| β-catenin | 17C2 (monoclonal) | Novo-castra | EDTA | 1:20 | 45 | Normal breast |
| E-cadherin (extracellular domain) | 36B5 (monoclonal) | Novo-castra | Citrate | 1:20 | 45 | Normal prostate |
| E-cadherin (cytoplasmic domain) | 36/E-cadherin (monoclonal) | BD Biosciences | EDTA | 1:100 | 45 | Normal skin |
| DVL1 | ab21062 (polyclonal) | Abcam | EDTA | 1:250 | 45 | Breast carcinoma |
| Her2/neu | AO485 (polyclonal) | Dako | EDTA | 1:200 | 45 | Breast carcinoma |

A negative control, in which the primary antibody was replaced by PBS (buffer), was run simultaneously for each antibody.

### 2.4.3 Immunohistochemistry

A representative formalin fixed paraffin embedded tissue block was retrieved for each case from the archive, after reviewing the initial haematoxylin and eosin (H&E) stained slides.

- Paraffin wax embedded sections were cut onto 3-aminopropyltriethoxysilane (APES) coated slides and heat-fixed overnight at 37°C to adhere sections to slides.
- Sections were dewaxed through xylene, rehydrated in graded ethanol and washed in water.
- Endogenous peroxidase activity was blocked by treating slides with a 1% hydrogen peroxide ($H_2O_2$) in water solution for 15 minutes.
- Slides were washed well in water.
- Antigen retrieval was performed by pressure-cooking slides in either citrate buffer at pH 6 for 2 minutes or EDTA (pH8) for 1 minute at full pressure.
- Slides were then immediately immersed in water.
- Slides were rinsed with phosphate buffered saline solution (PBS pH 7.6).
- Non-specific binding was blocked by treating slides with a 5% Goat Serum Solution (DAKO #X0907) at a concentration of 1:20
- Serum was then be drained off.
- Sections were incubated with primary antibody at room temperature at specified times and dilutions.
- Sections were washed well with PBS Buffer.
- Sections were incubated with DAKO Envision labelled Polymer, HRP (DAKO #K4001) for 30 minutes at room temperature.
- Sections were washed well with PBS buffer.
- Positivity was developed by applying the chromogenic substrate 3.3 – diaminobenzidine (DAKO K3466) for 5-10 minutes.
- Slides were washed in running tap water.
- Slides were immersed in a 1% copper sulphate ($CuSO_4$) solution for 5 minutes.
- Slides were washed in running tap water.

- Slides were counterstained in haematoxylin, blued in Scott's tap water.
- Slides were then washed in water, dehydrated using graded alcohols, cleared with xylene and mounted with rapid mountant medium (Entellan).

### 2.4.4 Interpretation of staining

β-catenin immunohistochemical staining was scored according to the protocol devised by Jass et al [92].

One point was allocated for loss of cell membrane staining; 1 for slight increase in cytoplasmic staining, 2 for marked increase in cytoplasmic staining; 1 for slight nuclear staining and 2 for pronounced nuclear staining. This tallies up to a maximum score of 5. According to this protocol, cases scoring 4 or more were regarded as positive for abnormal β-catenin immunolocalisation.

Dishevelled (DVL1), E-cadherin extracellular domain (36B5) and E-cadherin cytoplasmic domain (36/E-cadherin) immunohistochemical staining was scored according to the protocol used by Chetty et al. [93].

This scoring system regards only moderate and/or strong immunostaining as significant. The degree of positivity is quantified as follows:

- a score of 0 if less than or equal to 5% of tumour cells stained
- a score of 1 if 6% to 50% of tumour cells stained
- a score of 2 if more than 50% of tumour cells stained

Additionally, the cellular location of the immunoreactivity was noted.

For E-cadherin (extracellular domain) immunohistochemical stains, membranous or a combination of membranous and cytoplasmic staining represented normal E-cadherin localisation. Any deviation from this staining pattern (including absent staining) was considered immunohistochemically abnormal cellular localisation of the extracellular domain of E-cadherin.

For E-cadherin (cytoplasmic domain) immunohistochemical stains, cytoplasmic or a combination of membranous and cytoplasmic staining represented normal E-cadherin localisation. Any deviation from this staining pattern (including absent staining) was considered immunohistochemically abnormal cellular localisation of the cytoplasmic domain of E-cadherin

Her2/neu immunohistochemical staining was scored according to the criteria used in the ToGA trial [84]. This scoring system is summarised in Table 5.

Table 5: Criteria used in the ToGA trial for scoring Her2/neu expression by immunohistochemistry

| **Her2/neu score** | **Immunohistochemical staining pattern in surgical specimen** | **Her2/neu expression assessment** |
|---|---|---|
| 0 | No reactivity or membranous reactivity in <10% of tumour cells | Negative for overexpression by immunohistochemistry |
| 1+ | Faint/barely perceptible membranous reactivity in ≥ 10% of tumour cells; cells are reactive in only part of their membrane | Negative for overexpression by immunohistochemistry |
| 2+ | Weak to moderate complete, basolateral or lateral membranous reactivity in ≥ 10% of tumour cells | Equivocal for overexpression by immunohistochemistry |
| 3+ | Strong complete, basolateral or lateral membranous reactivity in ≥ 10% of tumour cells | Positive for overexpression by immunohistochemistry |

### 2.4.5 Tumour morphology

Gastric adenocarcinomas were defined as a primary malignant epithelial neoplasm demonstrating glandular differentiation, either through the formation of neoplastic glands or by the presence of discrete intracellular mucin.

The intestinal-type tumours were characterised by mitotically active epithelial cells with large nuclei, prominent nucleoli and intracytoplasmic mucin. The tumour cells formed glands, nests, sheets and tubules.

The diffuse-type tumours were characterised by linear cords of poorly cohesive or discohesive epithelial cells with moderate to severe nuclear hyperchromasia and minimal pale eosinophilic-to-clear cytoplasm. Signet-ring morphology was often apparent. Gland formation was inconspicuous. Occasional tumours showed small glands within the superficial regions of the neoplasm.

Mixed type tumours showed features of both intestinal and diffuse type tumour morphology.

### 2.4.6 Grading of tumours

Tumours were classified into well differentiated, moderately differentiated, poorly differentiated and undifferentiated carcinomas, based on the 2009 $7^{th}$ edition TNM tumour staging system [78].

According to this system, gastric carcinomas are graded based on the extent of glandular differentiation [Table 6].

Table 6: Tumour grading

| | | |
|---|---|---|
| Grade 1 | Well differentiated adenocarcinoma | Greater than 95% of the tumour is composed of glands |
| Grade 2 | Moderately differentiated adenocarcinoma | 50 – 95% of the tumour is composed of glands |
| Grade 3 | Poorly differentiated adenocarcinoma | 49% or less of the tumour is composed of glands |
| Grade 4 | Undifferentiated carcinoma | High grade carcinoma that cannot be further classified as adenocarcinoma, squamous cell carcinoma or any other recognised variants. |

### 2.4.7 Site of tumour

Tumour site was described as either proximal (involving cardia, fundus or body) or distal (involving antrum or pylorus). Information regarding the tumour site was indicated in the macroscopic descriptions in the pathology reports.

### 2.4.8 Interpretation of lymph node metastasis

The 2009 7th edition TNM tumour staging system [78] was used for evaluating lymph node metastases. Regional lymph nodes were described as positive for metastatic tumour if clusters of gastric adenocarcinoma cells, measuring more than 0.2mm in diameter, were present within the lymph node.

If no tumour cells, single tumour cells or isolated clusters of tumour cells measuring less than 0.2mm were noted within the lymph node, these were considered negative for nodal metastatic carcinoma.

Discontinuous tumour deposits located within the subserosal tissue adjacent to the gastric adenocarcinoma, where no evidence of a residual lymph node existed, were considered positive for nodal metastatic carcinoma.

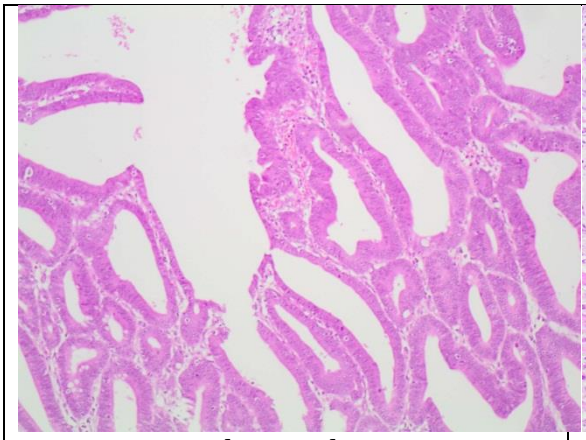
## 2.5 Statistical analysis

The Fisher's exact test, Shapiro-Wilk test, Bonferroni test, Chi squared test and Kappa test were used in the analysis of variables. Computerised statistical analysis and interpretation was performed using Stata 12.0 statistical software (StataCorp LP, College Station, USA).

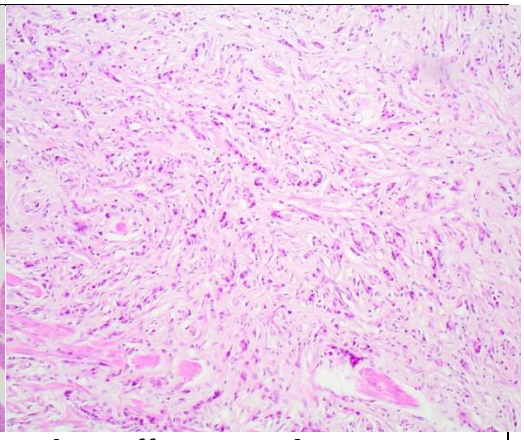
Results were considered statistically significant if $P < 0.05$

# 3.Results

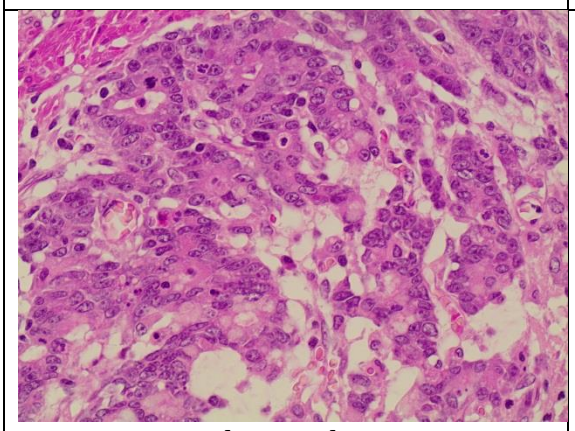
All cases were confirmed as gastric adenocarcinomas. There were 97 cases of gastric adenocarcinomas, comprising 39 intestinal type and 51 diffuse type gastric adenocarcinomas. 7 tumours showed mixed intestinal and diffuse morphology [Figure 2; Appendix 1].



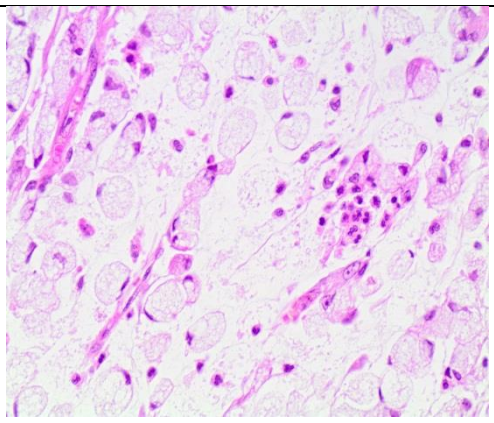
a. Intestinal-type adenocarcinoma (H&E, 10x obj mag)



b. Diffuse-type adenocarcinoma (H&E, 10x obj mag)



c. Intestinal-type adenocarcinoma (H&E, 40x obj mag)



d. Diffuse-type adenocarcinoma (H&E, 40x obj mag)

Figure 2: Photomicrographs of gastric adenocarcinoma. (a) Neoplastic cells forming nests and tubules. (b) Poorly cohesive cords of tumour cells present within a desmoplastic stroma. (c) Large tumour cells with eosinophilic cytoplasm, vesicular nuclei and prominent nucleoli. (d) Tumour cells with an eccentric nucleus displaced by intracytoplasmic vacuoles.

## 3.1 Age

Age was not provided on the request form or within the available clinical details in two cases (cases 34 and 42). These two cases were omitted from the analysis of age, thus resulting in 95 cases being statistically evaluated

The age range for all cases combined was 18 – 84 years, with a mean age of 57.1 years. Within the intestinal type adenocarcinomas, the age range varied from 32 – 84 years, with a mean age of 63.1 years. Diffuse type adenocarcinomas demonstrated an age range from 33 – 82 years, with a mean age of 54.5 years. Gastric adenocarcinomas showing mixed morphology had an age range from 18 – 57 years, with a mean age of 45 years [Table 7].

Table 7: Comparison of age (years) between gastric adenocarcinoma groups

| | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| Number of cases | 95 | 37 | 51 | 7 |
| Age range (years) | 18 - 84 | 32 - 84 | 33 - 82 | 18 – 57 |
| Mean age +/- SD (years) | 57.1 +/- 13.7 | 63.1 +/- 12.9 | 54.5 +/- 12.5 | 45 +/- 12.9 |

Statistically significant differences were noted when comparing age amongst intestinal and diffuse gastric adenocarcinoma subtypes (P = 0.007) and between intestinal and mixed subtypes (P = 0.002) [Table 8].

Table 8: Comparison of age by tumour type (Bonferroni test).

| | Intestinal type | Diffuse type |
|---|---|---|
| Diffuse type | -8.61791<br>P = 0.007 | |
| Mixed type | -18.1081<br>P = 0.002 | -9.4902<br>P = 0.200 |

## 3.2 Gender

Gender information was provided on the request forms of all 97 cases. [Table 9]

Table 9: Comparison of gender between gastric adenocarcinoma groups

| | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| Number of cases | 97 | 39 | 51 | 7 |
| Male (%) | 51 (53%) | 24 (62%) | 22 (43%) | 3 (43%) |
| Female (%) | 48 (47%) | 15 (38%) | 29 (57%) | 4 (57%) |
| Fisher's exact test | | P = 0.271 | | |

When comparing all cases of gastric carcinomas, more males than females were afflicted. Intestinal type gastric adenocarcinomas were noted in more males (62%) than females (38%), while diffuse type and mixed type tumours showed a female predominance (57% each). However, no statistically significant differences were present in the comparison of the tumour subtypes by gender.

## 3.3 Tumour differentiation

Sixty seven percent (67%) of intestinal type tumours were well or moderately differentiated while 100% of diffuse type carcinomas were poorly differentiated. All cases of tumours with mixed intestinal and diffuse morphology (mixed subtype), were poorly differentiated. A significantly higher proportion of diffuse type tumours were poorly differentiated ($P < 0.0001$) when compared to intestinal type tumours. A significantly greater proportion of mixed type tumours showed poor differentiation when compared to intestinal type tumours [Tables 10 - 13].

Table 10: Comparison of tumour differentiation between gastric adenocarcinoma groups

| | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| Well differentiated | 2 (1%) | 2 (5%) | 0 (0%) | 0 (0%) |
| Moderately differentiated | 26 (27%) | 26 (67%) | 0 (0%) | 0 (0%) |
| Poorly differentiated | 69 (72%) | 11 (28%) | 51 (100%) | 7 (100%) |
| Total | 97 (100%) | 39 (40%) | 51 (53%) | 7 (7%) |

Table 11: Comparison of tumour differentiation between intestinal and diffuse type tumours

| | Intestinal type | Diffuse type |
|---|---|---|
| Well or moderately differentiated | 28 cases | 0 cases |
| Poorly differentiated | 11 cases | 51 cases |
| Chi-squared test | $P < 0.0001$ | |

Table 12: Comparison of tumour differentiation between intestinal and mixed type tumours

| | Intestinal type | Mixed type |
|---|---|---|
| Well or moderately differentiated | 28 cases | 0 cases |
| Poorly differentiated | 11 cases | 7 cases |
| Chi-squared test | P = 0.0004 | |

Table 13: Comparison of tumour differentiation between diffuse and mixed type tumours

| | Diffuse type | Mixed type |
|---|---|---|
| Well or moderately differentiated | 0 cases | 0 cases |
| Poorly differentiated | 51 cases | 7 cases |
| Chi-squared test | P = 1.0000 | |

## 3.4 Depth of invasion

The majority of intestinal type carcinomas showed invasion into the muscularis propria or subserosa (pT2). This contrasted with more extensive serosal (pT3) tumour invasion noted in diffuse type adenocarcinomas. Mixed type tumours showed an equal proportion of pT2 and pT3 invasion [Table 14].

No statistically significant differences were present in comparison of the minimal–to-moderately invasive (pT1/pT2) tumours to deeply invasive (pT3/pT4) tumours amongst the tumour subtypes [Tables 15-17].

Table 14: Comparison of tumour depth of invasion between gastric adenocarcinoma groups

| | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| pT1 | 12 (12%) | 4 (10%) | 8 (16%) | 0 (0%) |
| pT2 | 42 (44%) | 20 (51%) | 19 (37%) | 3 (43%) |
| pT3 | 40 (41%) | 15 (39%) | 22 (43%) | 3 (43%) |
| pT4 | 3 (3%) | 0 (0%) | 2 (4%) | 1 (14%) |
| Total | 97 (100%) | 39 (40%) | 51 (53%) | 7 (7%) |

Table 15: Comparison of tumour depth of invasion between intestinal and diffuse type tumours:

| | Intestinal type | Diffuse type |
|---|---|---|
| pT1 or pT2 | 24 cases | 27 cases |
| pT3 or pT4 | 15 cases | 24 cases |
| Chi squared test | P = 0.4147 | |

Table 16: Comparison of tumour depth of invasion between intestinal and mixed type tumours:

| | Intestinal type | Mixed type |
|---|---|---|
| pT1 or pT2 | 24 cases | 3 cases |
| pT3 or pT4 | 15 cases | 4 cases |
| Chi squared test | P = 0.3553 | |

Table 17: Comparison of tumour depth of invasion between diffuse and mixed type tumours:

| | Diffuse type | Mixed type |
|---|---|---|
| pT1 or pT2 | 27 cases | 3 cases |
| pT3 or pT4 | 24 cases | 4 cases |
| Chi squared test | P = 0.6166 | |

## 3.5 Site of tumour

Most tumours had a proximal distribution (57%), with all tumour groups showing a proximal predominance. No statistically significant differences between tumour location and tumour subtypes were present. [Table 18]

Table 18: Comparison of tumour site between gastric adenocarcinoma groups

| | Intestinal type | Diffuse type | Mixed type | Total no. of cases |
|---|---|---|---|---|
| Proximal tumour location | 21 (54%) | 29 (57%) | 5 (71%) | 55 |
| Distal tumour location | 18 (46%) | 22 (43%) | 2 (29%) | 42 |
| Fisher's exact test<br>P = 0.7753 | | | | |

## 3.6 Nodal metastases

Lymph nodes were recovered in 94 of the 97 gastrectomy specimens. All tumour subtypes showed regional lymph node metastases, detected in 62% of intestinal type, 61% of diffuse type and 85% of mixed tumours respectively. In the 94 cases where lymph nodes were recovered, no statistically significant differences in regional lymph node metastases were present amongst the tumour subtypes. [Tables 19-22]

Table 19: Comparison of regional lymph node metastases between gastric adenocarcinoma groups.

| | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| Positive for nodal metastasis | 61 (63%) | 24 (62%) | 31 (61%) | 6 (85%) |
| Negative for nodal metastasis | 33 (34%) | 13 (33%) | 19 (37%) | 1 (15%) |
| No lymph nodes present | 3 (3%) | 2 (5%) | 1 (2%) | 0 (0%) |

Table 20: Comparison of regional lymph node metastases between intestinal and diffuse type tumours

| | Intestinal type | Diffuse type |
|---|---|---|
| Positive for nodal metastasis | 24 (62%) | 31 (61%) |
| Negative for nodal metastasis | 13 (33%) | 19 (37%) |
| Chi squared test | P = 0.7841 | |

Table 21: Comparison of regional lymph node metastases between intestinal and mixed type tumours

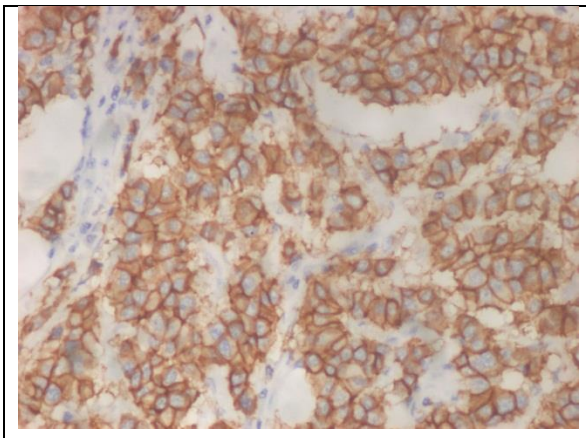
| | Intestinal type | Mixed type |
|---|---|---|
| Positive for nodal metastasis | 24 (62%) | 6 (85%) |
| Negative for nodal metastasis | 13 (33%) | 1 (15%) |
| Chi squared test | P = 0.2771 | |

Table 22: Comparison of regional lymph node metastases between diffuse and mixed type tumours

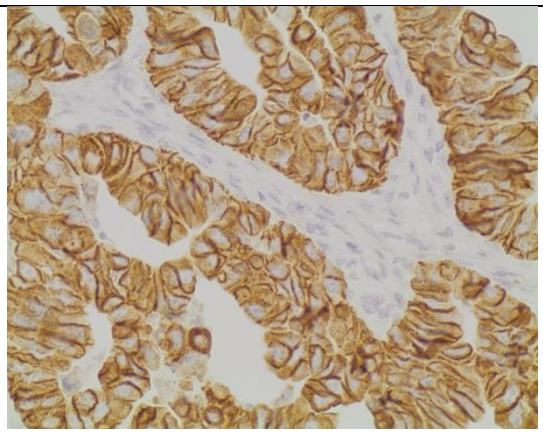
| | Diffuse type | Mixed type |
|---|---|---|
| Positive for nodal metastasis | 31 (61%) | 6 (85%) |
| Negative for nodal metastasis | 19 (37%) | 1 (15%) |
| Chi squared test | P = 0.2182 | |

## 3.7 Her2/neu

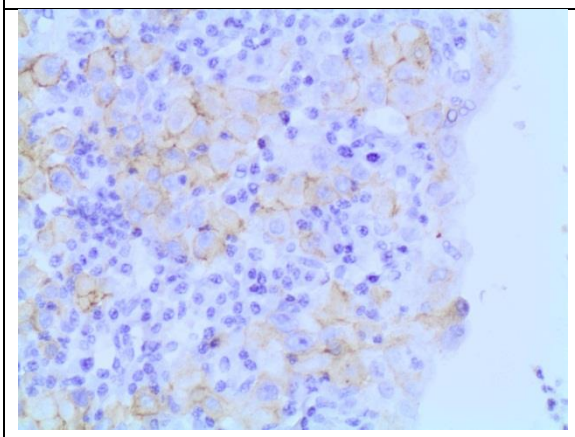
Her2/neu immunohistochemical staining was performed on all 97 cases [Figure 3].



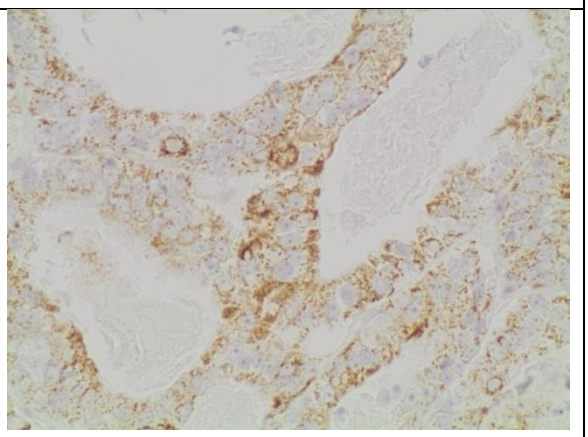
a. External control (Her2/neu, 20x obj mag)



b. Intestinal type gastric adenocarcinoma score = 3+ (Her2/neu, 20x obj mag)



c. Diffuse gastric adenocarcinoma score = 1+ (Her2/neu, 20x obj mag)



d. Intestinal type gastric adenocarcinoma granular staining (Her2/neu, 20x obj mag)

Figure 3. (a) Her2/neu breast carcinoma positive external control showing complete strong membrane staining. (b) Gastric adenocarcinoma (intestinal type) Her2/neu showing complete strong membranous staining in 100% of tumour cells. (c) Diffuse gastric adenocarcinoma Her2/neu showing faint barely perceptible membranous staining. (d) Non-specific granular cytoplasmic staining in intestinal type gastric adenocarcinoma epithelial cells.

Twelve (12) of the 97 cases demonstrated 3+ immunopositivity and thus were considered to overexpress Her2/neu. A statistically significant proportion (P = 0.0174) of intestinal type tumours (23%) showed Her2/neu overexpression when compared to diffuse type tumours (6%). [Table 25]

A single case showed non-specific granular cytoplasmic Her2/neu staining. This staining did not represent overexpression.

Table 23: Distribution of Her2/neu staining between gastric adenocarcinoma groups.

| Her2/neu score | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| 0 | 15 (15%) | 4 (10%) | 9 (18%) | 2 (29%) |
| 1+ | 57 (60%) | 20 (51%) | 33 (64%) | 4 (57%) |
| 2+ | 12 (12%) | 5 (13%) | 6 (12%) | 1 (14%) |
| 3+ | 12 (12%) | 9 (23%) | 3 (6%) | 0 (0%) |
| Granular cytoplasmic | 1 (%) | 1 (3%) | 0 (0%) | 0 (0%) |
| Total | 97 (100%) | 39 (40%) | 51 (53%) | 7 (7%) |

Table 24: Comparison of Her2/neu immunolocalisation between gastric adenocarcinoma groups.

| | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| Positive for Her2/neu overexpression | 12 (12%) | 9 (23%) | 3 (6%) | 0 (0%) |
| Negative/equivocal for Her2/neu overexpression | 85 (88%) | 30 (77%) | 48 (94%) | 7 (100%) |
| Total | 97 (100%) | 39 (40%) | 51 (53%) | 7 (7%) |

Table 25: Comparison of Her2/neu immunolocalisation between intestinal and diffuse gastric adenocarcinoma groups.

| | Intestinal type | Diffuse type |
|---|---|---|
| Positive for Her2/neu overexpression | 9 cases | 3 cases |
| Negative/equivocal for Her2/neu overexpression | 30 cases | 48 cases |
| Chi squared test | P = 0.0174 | |

Table 26: Comparison of Her2/neu immunolocalisation between intestinal and mixed gastric adenocarcinoma groups.

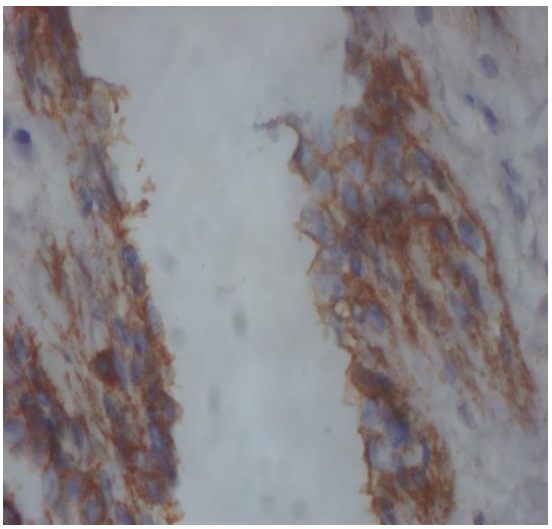
| | Intestinal type | Mixed type |
|---|---|---|
| Positive for Her2/neu overexpression | 9 cases | 0 cases |
| Negative/equivocal for Her2/neu overexpression | 30 cases | 7 cases |
| Chi squared test | P = 0.1564 | |

Table 27: Comparison of Her2/neu immunolocalisation between diffuse and mixed gastric adenocarcinoma groups.

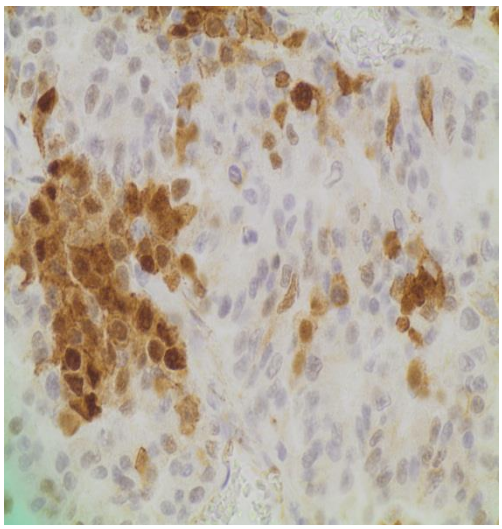
| | Diffuse type | Mixed type |
|---|---|---|
| Positive for Her2/neu overexpression | 3 cases | 0 cases |
| Negative/equivocal for Her2/neu overexpression | 48 cases | 7 cases |
| Chi squared test | P = 0.5099 | |

## 3.8 β-Catenin

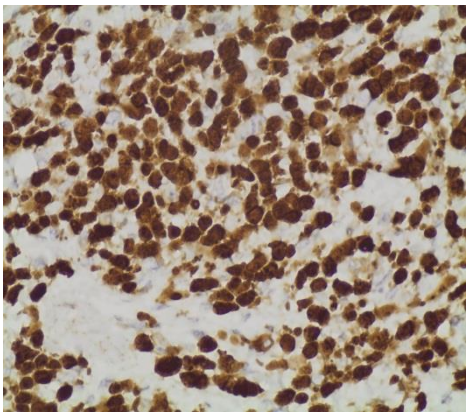
Immunohistochemical staining for β-catenin was performed on all 97 cases of gastric adenocarcinomas. [Figure 4]



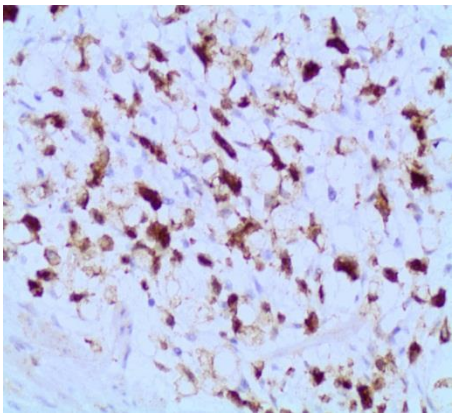
a. External control (β-catenin, 40x obj mag)



b. β-catenin score = 4 (intestinal type gastric adenocarcinoma), 40x obj mag



c. β-catenin score = 3 (diffuse type gastric adenocarcinoma), 40x obj mag



d. β-catenin score = 5 (diffuse type gastric adenocarcinoma), 40x obj mag

Figure 4. (a) β-catenin positive external control showing membrane staining in normal breast ducts. (b) Moderate cytoplasmic and strong nuclear β-catenin staining with loss of membrane staining, equating to a score of 4 in an intestinal-type gastric adenocarcinoma. (c) Strong nuclear β-catenin staining with loss of membrane staining, equating to a score of 3 in a diffuse type gastric adenocarcinoma. (d) Strong nuclear and cytoplasmic β-catenin staining with loss of membrane staining, equating to a score of 5 in a diffuse type gastric adenocarcinoma.

Fourteen (14) of the 97 gastric adenocarcinoma cases (14%) showed abnormal localisation of β-catenin as detected by immunohistochemistry. Abnormal β-catenin immunolocalisation was noted in all three gastric adenocarcinoma subgroups, occurring in 13%, 16% and 14% of intestinal type, diffuse type and mixed type tumours, respectively [Table 28].

No statistically significant difference in β-catenin immunolocalisation amongst the tumour subgroups was evident [Tables 29-31].

Table 28: Comparison of abnormal β-catenin immunolocalisation between gastric adenocarcinoma groups.

| | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| Abnormal localisation | 14 (14%) | 5 (13 %) | 8 (16%) | 1 (14%) |
| No abnormality detected | 83 (86%) | 34 (87%) | 43 (84%) | 6 (86%) |

Table 29: Comparison of abnormal β-catenin immunolocalisation between intestinal and diffuse type tumours

| | Intestinal type | Diffuse type |
|---|---|---|
| Abnormal localisation | 5 cases | 8 cases |
| No abnormality detected | 34 cases | 43 cases |
| Chi squared test | P = 0.7015 | |

Table 30: Comparison of abnormal β-catenin immunolocalisation between intestinal and mixed type tumours

| | Intestinal type | Mixed type |
|---|---|---|
| Abnormal localisation | 5 cases | 1 case |
| No abnormality detected | 34 cases | 6 cases |
| Chi squared test | P = 0.9156 | |

Table 31: Comparison of abnormal β-catenin immunolocalisation between diffuse and mixed type tumours

| | Diffuse type | Mixed type |
|---|---|---|
| Abnormal localisation | 8 cases | 1 case |
| No abnormality detected | 43 cases | 6 cases |
| Chi squared test | P = 0.9235 | |

## 3.9 Her2/neu and β-Catenin

No significant correlations were noted in the comparison of aberrant β-catenin immunohistochemical localisation and Her2/neu overexpression amongst the different groups of adenocarcinomas. Only a single case showed a combination of Her2/neu overexpression and aberrant β-catenin immunohistochemical localisation [Tables 32-35].

Table 32: Correlation between Her2/neu and β-Catenin staining in all gastric adenocarcinomas.

| | Her2/neu overexpressed | No Her2/neu overexpression |
|---|---|---|
| Normal β-Catenin localisation | 11 cases | 72 cases |
| Abnormal β-Catenin localisation | 1 case | 13 cases |
| Chi squared test | P = 0.5206 | |

Table 33: Correlation between Her2/neu and β-Catenin staining in intestinal type gastric adenocarcinomas.

| | Her2/neu overexpressed | No Her2/neu overexpression |
|---|---|---|
| Normal β-Catenin localisation | 9 cases | 25 cases |
| Abnormal β-Catenin localisation | 0 case | 5 cases |
| Chi squared test | P = 0.1896 | |

Table 34: Correlation between Her2/neu and β-Catenin staining in diffuse type gastric adenocarcinomas.

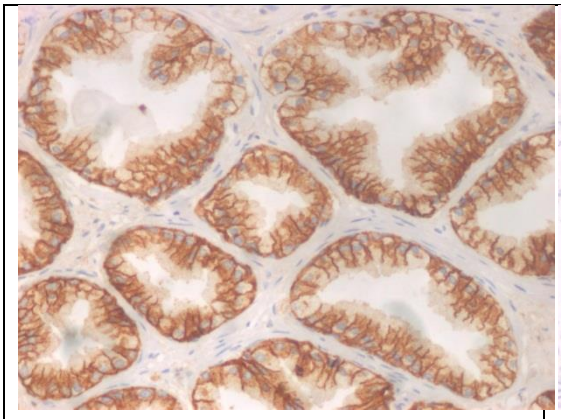
| | Her2/neu overexpressed | No Her2/neu overexpression |
|---|---|---|
| Normal β-Catenin localisation | 2 cases | 41 cases |
| Abnormal β-Catenin localisation | 1 case | 7 cases |
| Chi squared test | P = 0.3863 | |

Table 35: Correlation between Her2/neu and β-Catenin staining in mixed type gastric adenocarcinomas.

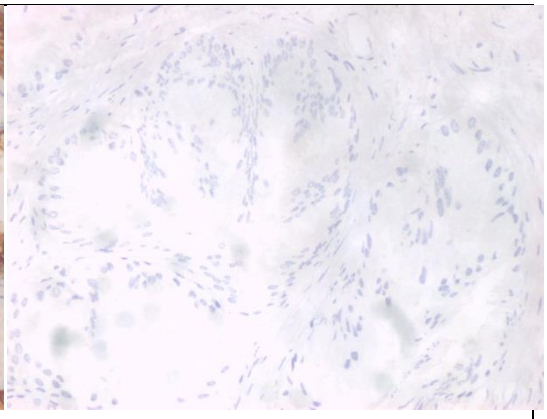
| | Her2/neu overexpressed | No Her2/neu overexpression |
|---|---|---|
| Normal β-Catenin localisation | 0 cases | 6 cases |
| Abnormal β-Catenin localisation | 0 case | 1 cases |
| Chi squared test | P = 1.0000 | |

## 3.10 E-cadherin (extracellular domain)

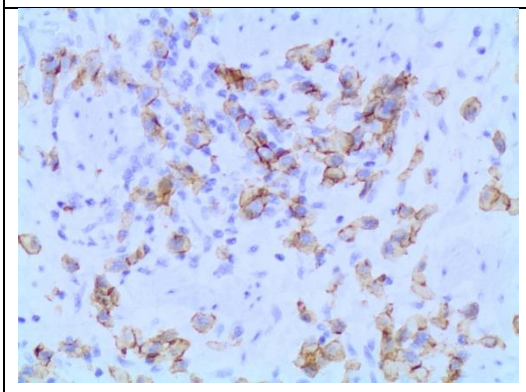
All 97 cases were immunohistochemically stained to detect the extracellular domain of E-cadherin [Figure 5].



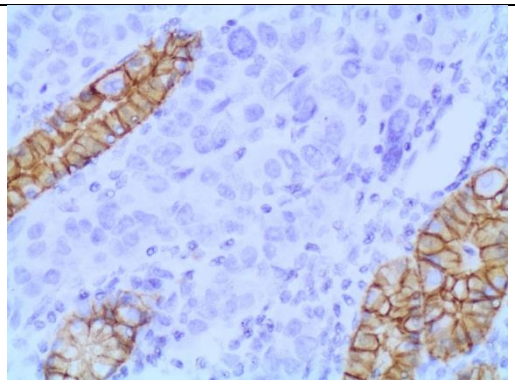
a. Positive external control (E-cadherin extracellular domain, 20x obj mag)



b. Negative external control (E-cadherin extracellular domain, 20x obj mag)



c. E-cadherin extracellular domain, 40x obj mag)



d. E-cadherin extracellular domain, 40x obj mag)

Figure 5. (a) E-cadherin (extracellular domain) positive external control showing strong membranous staining in prostatic gland epithelium. (b) E-cadherin (extracellular domain) negative external control showing no staining of prostatic gland epithelium. (c) E-cadherin (extracellular domain) showing strong membrane staining in a diffuse type tumour. (d) E-cadherin (extracellular domain) showing absent staining in a diffuse type tumour (with positive internal control)

Seven cases (18%) of intestinal type tumours showed abnormal cytoplasmic localisation of E-cadherin, while being seen in 29 cases (57%) of diffuse type tumours. Abnormal E-cadherin localisation was manifested by absent staining in 4 cases (8%) and cytoplasmic staining in 25 cases (49%). All mixed type tumours showed normal E-cadherin localisation [Table 36].

Comparing intestinal and diffuse type tumour subgroups, a significantly greater proportion of diffuse type tumours ($P < 0.001$) showed abnormal E-cadherin immunolocalisation [Table 37]. A significantly greater proportion ($P = 0.0047$) of diffuse type tumours showed abnormal E-cadherin immunolocalisation when compared to mixed type tumours [Table 39].

Table 36: Comparison of E-cadherin (extracellular domain) immunolocalisation between gastric adenocarcinoma groups.

| | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| Normal localisation | 61 (63%) | 32 (82%) | 22 (43%) | 7 (100%) |
| Abnormal localisation | 36 (37%) | 7 (18%) | 29 (57%) | 0 (0%) |
| Total | 97 (100%) | 39 (40%) | 51 (53%) | 7 (7%) |

Table 37: Comparison of immunolocalisation of E-cadherin (extracellular domain) in intestinal and diffuse type tumours.

| | Intestinal type | Diffuse type |
|---|---|---|
| Normal immunolocalisation | 32 cases | 22 cases |
| Abnormal immunolocalisation | 7 cases | 29 cases |
| Chi squared test | $P < 0.001$ | |

Table 38: Comparison of immunolocalisation of E-cadherin (extracellular domain) in intestinal and mixed type tumours.

| | Intestinal type | Mixed type |
|---|---|---|
| Normal immunolocalisation | 32 cases | 7 cases |
| Abnormal immunolocalisation | 7 cases | 0 cases |
| Chi squared test | P = 0.2234 | |

Table 39: Comparison of immunolocalisation of E-cadherin (extracellular domain) in diffuse and mixed type tumours.

| | Diffuse type | Mixed type |
|---|---|---|
| Normal immunolocalisation | 22 cases | 7 cases |
| Abnormal immunolocalisation | 29 cases | 0 cases |
| Chi squared test | P = 0.0047 | |

Table 40: Distribution of E-cadherin (extracellular domain) staining between gastric adenocarcinoma groups.

| E-cadherin staining | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| No staining | 4 (4%) | 0 (0%) | 4 (8%) | 0 (0%) |
| Membranous | 37 (38%) | 22 (56%) | 8 (16%) | 7 (100%) |
| Membranous & cytoplasmic | 24 (25%) | 10 (26%) | 14 (27%) | 0 (0%) |
| Cytoplasmic | 32 (33%) | 7 (18 %) | 25 (49%) | 0 (0%) |
| Nuclear & cytoplasmic | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Nuclear | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Nuclear & Membranous | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Total | 97 (100%) | 39 (40%) | 51 (53%) | 7 (7%) |

Comparing the proportion of tumour cells showing immunopositive staining between intestinal and diffuse tumours, a significantly greater proportion of diffuse type tumours ($P = 0.0011$) showed staining in $\leq 5\%$ of tumour cells compared to intestinal type tumours [Table 42].

Table 41: Comparison of proportion of E-cadherin (extracellular domain) staining between gastric adenocarcinoma groups.

| Proportion of tumour cells staining | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| 0 (≤5%) | 12 (12%) | 0 (0%) | 12 (24%) | 0 (0%) |
| 1 (6 – 50%) | 3 (3%) | 1 (3%) | 2 (4%) | 0 (0%) |
| 2 (>50%) | 82 (85%) | 38 (97%) | 37 (73%) | 7 (100%) |
| Total | 97 (100%) | 39 (40%) | 51 (53%) | 7 (7%) |

Table 42: Comparison of proportion of E-cadherin (extracellular domain) staining between intestinal and diffuse tumours

| | Intestinal type | Diffuse type |
|---|---|---|
| ≤5% tumour cells staining | 0 cases | 12 cases |
| > 5% tumour cells staining | 39 cases | 39 cases |
| Chi squared test | P = 0.0011 | |

Table 43: Comparison of proportion of E-cadherin (extracellular domain) staining between intestinal and mixed tumours

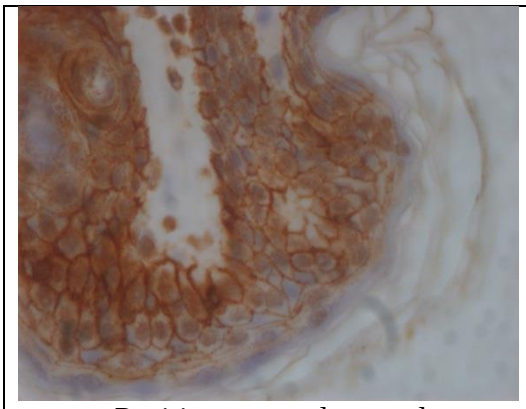
| | Intestinal type | Mixed type |
|---|---|---|
| ≤5% tumour cells staining | 0 cases | 0 cases |
| > 5% tumour cells staining | 39 cases | 7 cases |
| Chi squared test | P = 1.000 | |

Table 44: Comparison of proportion of E-cadherin (extracellular domain) staining between diffuse and mixed tumours

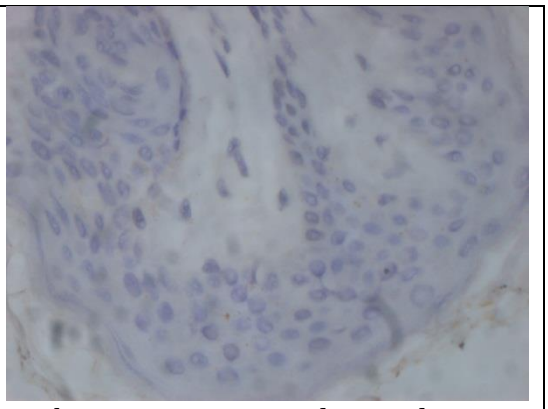
| | Diffuse type | Mixed type |
|---|---|---|
| ≤5% tumour cells staining | 12 cases | 0 cases |
| > 5% tumour cells staining | 39 cases | 7 cases |
| Chi squared test | P = 0.1496 | |

## 3.11 E-cadherin (cytoplasmic domain)

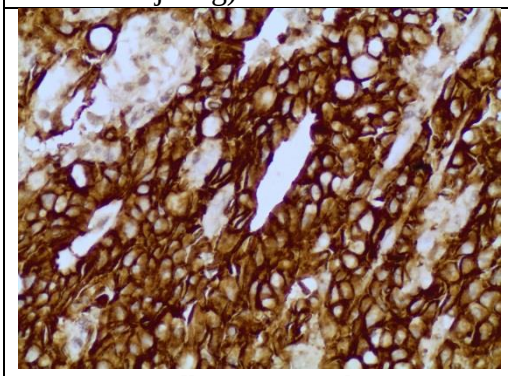
All 97 cases were immunohistochemically stained to detect the cytoplasmic domain of E-cadherin [Figure 6].



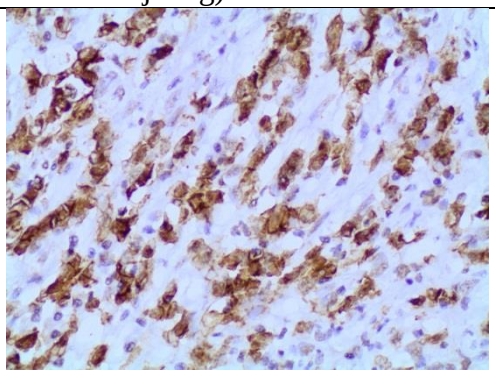
a. Positive external control (E-cadherin cytoplasmic domain, 40x obj mag)



b. Negative external control (E-cadherin cytoplasmic domain, 40x obj mag)



c. E-cadherin (cytoplasmic domain, 40X obj mag)



d. E-cadherin (cytoplasmic domain, 40X obj mag)

Figure 6. (a) E-cadherin (cytoplasmic domain) positive external control showing membranous and cytoplasmic staining in keratinocytes of normal skin. (b) E-cadherin (cytoplasmic domain) negative external control showing no staining of skin keratinocytes. (c) E-cadherin (cytoplasmic domain) showing strong membrane and cytoplasmic staining in an intestinal type gastric adenocarcinoma. (d) E-cadherin (cytoplasmic domain) showing strong cytoplasmic staining in a diffuse type gastric adenocarcinoma.

Eight cases of the 97 gastric adenocarcinomas (8%) showed abnormal E-cadherin localisation, with 6 cases (12%) noted within diffuse type adenocarcinomas and 2 cases (5%) noted within intestinal type adenocarcinomas. No adenocarcinomas with mixed morphology demonstrated abnormal E-cadherin localisation. [Table 45]

No statistically significant differences were present in either E-cadherin immunolocalisation or proportion of positive tumour cells between the tumour subgroups.

Table 45: Comparison of E-cadherin (cytoplasmic domain) immunolocalisation between gastric adenocarcinoma groups.

| | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| Normal localisation | 89 (92%) | 37 (95%) | 45 (88%) | 7 (100%) |
| Abnormal localisation | 8 (8%) | 2 (5%) | 6 (12%) | 0 (0%) |
| Total | 97 (100%) | 39 (40%) | 51 (53%) | 7 (7%) |

Table 46: Comparison of E-cadherin (cytoplasmic domain) immunolocalisation between intestinal and diffuse type tumours.

| | Intestinal type | Diffuse type |
|---|---|---|
| Normal localisation | 37 cases | 45 cases |
| Abnormal localisation | 2 cases | 6 cases |
| Chi squared test | P = 0.2729 | |

Table 47: Comparison of E-cadherin (cytoplasmic domain) immunolocalisation between intestinal and mixed type tumours.

| | Intestinal type | Mixed type |
|---|---|---|
| Normal localisation | 37 cases | 7 cases |
| Abnormal localisation | 2 cases | 0 cases |
| Chi squared test | P = 0.5401 | |

Table 48: Comparison of E-cadherin (cytoplasmic domain) immunolocalisation between diffuse and mixed type tumours.

| | Diffuse type | Mixed type |
|---|---|---|
| Normal localisation | 45 cases | 7 cases |
| Abnormal localisation | 6 cases | 0 cases |
| Chi squared test | P = 0.3378 | |

Table 49: Distribution of E-cadherin (cytoplasmic domain) staining between gastric adenocarcinoma groups.

| E-cadherin staining | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| No staining | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Membranous | 1 (1%) | 1 (3%) | 0 (0%) | 0 (0%) |
| Membranous & cytoplasmic | 68 (70%) | 27 (68%) | 37 (73%) | 4 (57%) |
| Cytoplasmic | 21 (22%) | 10 (26%) | 8 (16%) | 3 (43%) |
| Nuclear & cytoplasmic | 4 (4%) | 1 (3%) | 3 (6%) | 0 (0%) |
| Nuclear | 2 (2%) | 0 (0%) | 2 (4%) | 0 (0%) |
| Nuclear & Membranous | 1 (1%) | 0 (0%) | 1 (1%) | 0 (0%) |
| Total | 97 (100%) | 39 (40%) | 51 (53%) | 7 (7%) |

Table 50: Comparison of proportion of E-cadherin (cytoplasmic domain) staining between gastric adenocarcinoma groups.

| Proportion of tumour cells staining | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| 0 (<5%) | 1 (1%) | 0 (0%) | 1 (2%) | 0 (0%) |
| 1 (6 – 50%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| 2 (>50%) | 96 (99%) | 39 (100%) | 50 (98%) | 7 (100%) |
| Total | 97 (100%) | 39 (40%) | 51 (53%) | 7 (7%) |
| Fisher's exact test | P = 1.0000 | | | |

## 3.12 E-cadherin cytoplasmic and extracellular domains

There was a statistically significant correlation (P = 0.00207) amongst the combined group of tumour subtypes regarding abnormal immunolocalisation of the E-cadherin cytoplasmic domain and the E-cadherin membranous domain. [Table 51] In addition, a statistically significant (P = 0.02313) proportion of diffuse type tumours showed abnormal immunolocalisation of both E-cadherin clones. [Table 53] Intestinal and mixed type tumours showed no significant correlations regarding immunolocalisation of the two E-cadherin domains.

Table 51: Correlation between E-cadherin (cytoplasmic domain) and E-cadherin (extracellular domain) staining in all gastric adenocarcinomas.

| | Normal E-cadherin (cytoplasmic domain) localisation | Abnormal E-cadherin (cytoplasmic domain) localisation |
|---|---|---|
| Normal E-cadherin (extracellular domain) localisation | 60 cases | 1 cases |
| Abnormal E-cadherin (extracellular domain) localisation | 29 cases | 7 cases |
| Chi squared test | P = 0.00207 | |

Table 52: Correlation between E-cadherin (cytoplasmic domain) and E-cadherin (extracellular domain) staining in intestinal type gastric adenocarcinomas.

| | Normal E-cadherin (cytoplasmic domain) localisation | Abnormal E-cadherin (cytoplasmic domain) localisation |
|---|---|---|
| Normal E-cadherin (extracellular domain) localisation | 31 cases | 1 case |
| Abnormal E-cadherin (extracellular domain) localisation | 6 cases | 1 case |
| Chi squared test | P = 0.22527 | |

Table 53: Correlation between E-cadherin (cytoplasmic domain) and E-cadherin (extracellular domain) staining in diffuse type gastric adenocarcinomas.

| | Normal E-cadherin (cytoplasmic domain) localisation | Abnormal E-cadherin (cytoplasmic domain) localisation |
|---|---|---|
| Normal E-cadherin (extracellular domain) localisation | 22 cases | 0 cases |
| Abnormal E-cadherin (extracellular domain) localisation | 23 cases | 6 cases |
| Chi squared test | P = 0.02313 | |

Table 54: Correlation between E-cadherin (cytoplasmic domain) and E-cadherin (extracellular domain) staining in mixed type gastric adenocarcinomas.

| | Normal E-cadherin (cytoplasmic domain) localisation | Abnormal E-cadherin (cytoplasmic domain) localisation |
|---|---|---|
| Normal E-cadherin (extracellular domain) localisation | 7 cases | 0 cases |
| Abnormal E-cadherin (extracellular domain) localisation | 0 cases | 0 cases |
| Chi squared test | P = 1.0000 | |

## 3.13 E-cadherin (cytoplasmic domain) and β-catenin

There was a statistically significant (P = 0.0139) correlation between abnormal immunolocalisation of E-cadherin (cytoplasmic domain) and abnormal immunolocalisation of β-Catenin amongst diffuse type gastric adenocarcinomas. [Table 57] Intestinal and mixed type tumours showed no such correlations.

Table 55: Correlation between E-cadherin (cytoplasmic domain) and β-Catenin staining in all gastric adenocarcinomas.

| | Normal E-cadherin localisation | Abnormal E-cadherin localisation |
|---|---|---|
| Normal β-Catenin localisation | 78 cases | 5 cases |
| Abnormal β-Catenin localisation | 11 cases | 3 cases |
| Chi squared test | P = 0.0526 | |

Table 56: Correlation between E-cadherin (cytoplasmic domain) and β-Catenin staining in intestinal type gastric adenocarcinomas.

| | Normal E-cadherin localisation | Abnormal E-cadherin localisation |
|---|---|---|
| Normal β-Catenin localisation | 32 cases | 2 cases |
| Abnormal β-Catenin localisation | 5 cases | 0 cases |
| Chi squared test | P = 0.57767 | |

Table 57: Correlation between E-cadherin (cytoplasmic domain) and β-Catenin staining in diffuse type gastric adenocarcinomas.

| | Normal E-cadherin localisation | Abnormal E-cadherin localisation |
|---|---|---|
| Normal β-Catenin localisation | 40 cases | 3 cases |
| Abnormal β-Catenin localisation | 5 cases | 3 cases |
| Chi squared test | P = 0.0139 | |

Table 58: Correlation between E-cadherin (cytoplasmic domain) and β-Catenin staining in mixed type gastric adenocarcinomas.

| | Normal E-cadherin localisation | Abnormal E-cadherin localisation |
|---|---|---|
| Normal β-Catenin localisation | 6 cases | 0 cases |
| Abnormal β-Catenin localisation | 1 case | 0 cases |
| Chi squared test | P = 1.0000 | |

## 3.14 E-cadherin (extracellular domain) and β-catenin

No significant correlations were noted in the comparison of aberrant immunohistochemical localisation of the extracellular domain of E-cadherin and β-catenin amongst the different groups of gastric adenocarcinomas.

Table 59: Correlation between E-cadherin (extracellular domain) and β-Catenin staining in all gastric adenocarcinomas.

| | Normal E-cadherin localisation | Abnormal E-cadherin localisation |
|---|---|---|
| Normal β-Catenin localisation | 55 cases | 28 cases |
| Abnormal β-Catenin localisation | 6 cases | 8 cases |
| Chi squared test | P = 0.0935 | |

Table 60: Correlation between E-cadherin (extracellular domain) and β-Catenin staining in intestinal type gastric adenocarcinomas.

| | Normal E-cadherin localisation | Abnormal E-cadherin localisation |
|---|---|---|
| Normal β-Catenin localisation | 29 cases | 5 cases |
| Abnormal β-Catenin localisation | 3 cases | 2 cases |
| Chi squared test | P = 0.1688 | |

Table 61: Correlation between E-cadherin (extracellular domain) and β-Catenin staining in diffuse type gastric adenocarcinomas.

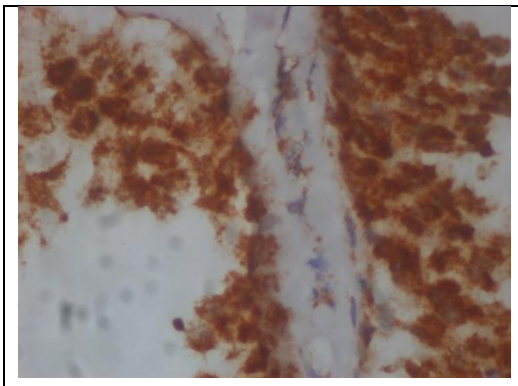
| | Normal E-cadherin localisation | Abnormal E-cadherin localisation |
|---|---|---|
| Normal β-Catenin localisation | 20 cases | 23 cases |
| Abnormal β-Catenin localisation | 2 cases | 6 cases |
| Chi squared test | P = 0.2593 | |

Table 62: Correlation between E-cadherin (extracellular domain) and β-Catenin staining in mixed type gastric adenocarcinomas.

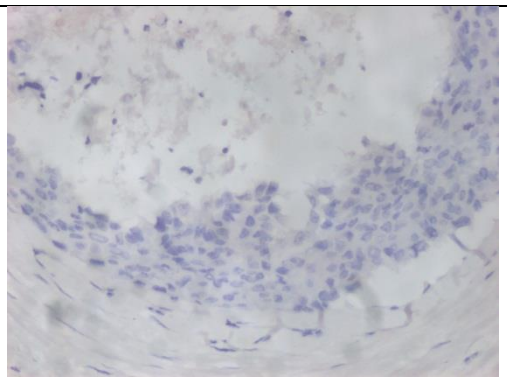
| | Normal E-cadherin localisation | Abnormal E-cadherin localisation |
|---|---|---|
| Normal β-Catenin localisation | 6 cases | 0 cases |
| Abnormal β-Catenin localisation | 1 case | 0 cases |
| Chi squared test | P = 1.0000 | |

## 3.15 Dishevelled

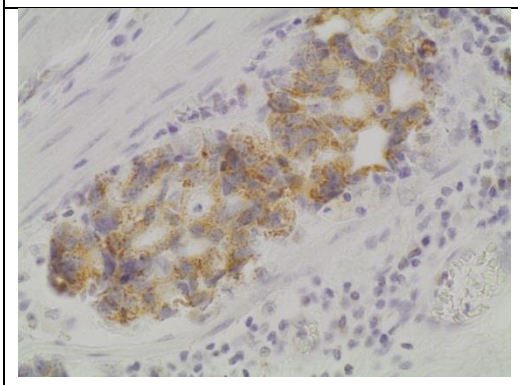
Immunohistochemical staining for Dishevelled was performed on all 97 cases of gastric adenocarcinoma [Figure 7].



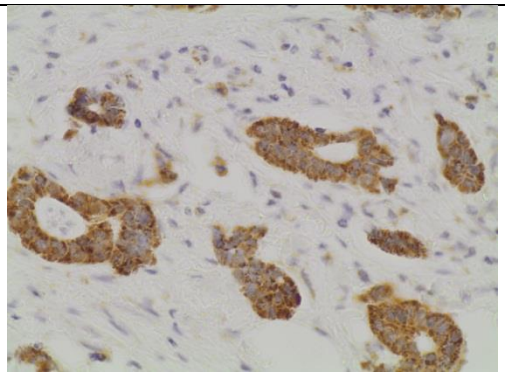
a. Positive external control (Dishevelled, 40x obj mag)



b. Negative external control (Dishevelled, 20x obj mag)



c. Intestinal type gastric adenocarcinoma score = 1 Dishevelled, 20x obj mag)



d. Intestinal type gastric adenocarcinoma score = 2 Dishevelled, 20x obj mag)

Figure 7. (a) Dishevelled positive external control showing strong cytoplasmic staining in breast carcinoma. (b) Dishevelled negative external control showing absent staining. (c) Moderate cytoplasmic staining in 6-50% of tumour cells. (d) Moderate cytoplasmic staining in more than 50% of tumour cells.

All cases demonstrated moderate to intense cytoplasmic staining in more than 5% of tumour cells. No statistically significant differences between the tumour subgroups were evident [Table 63].

Table 63: Comparison of proportion of Dishevelled staining between gastric adenocarcinoma groups.

| Proportion of tumour cells staining | All cases | Intestinal type | Diffuse type | Mixed type |
|---|---|---|---|---|
| 0 (<5%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| 1 (6 – 50%) | 9 (9%) | 4 (11%) | 3 (6%) | 2 (38%) |
| 2 (>50%) | 88 (91%) | 35 (89%) | 48 (94%) | 5 (72%) |
| Total | 97 (100%) | 39 (40%) | 51 (53%) | 7 (7%) |
| Fisher's exact test | | P = 0.134 | | |

## 3.16 Tumour analysis by age group (≤ 40 years and > 40 years)

Eleven cases (11% of the 95 cases analysed by age) of gastric adenocarcinoma were identified in patients 40 years and younger, consisting of 9 intestinal type tumours (82%), 1 diffuse type tumour (9%) and 1 mixed type tumour (9%) [Table 64].

No statistically significant difference (P = 0.0545) existed when comparing tumour type by age group.

Table 64: Comparison of tumour type by age group

| | ≤ 40 years old | > 40 years old |
|---|---|---|
| Intestinal type | 9 | 44 |
| Non intestinal type | 2 | 40 |
| Chi squared test | P = 0.0545 | |

Of the eleven cases in younger patients, 6 (54%) were in females and 5 (46%) were in males. This did not represent a significant finding (P = 06656) [Table 65].

Table 65: Gender comparison by age group

| | ≤ 40 years old | > 40 years old |
|---|---|---|
| Male | 5 cases | 44 |
| Female | 6 cases | 40 |
| Chi squared test | P = 06656 | |

One tumour (9%) was moderately differentiated and 10 tumours (91%) were poorly differentiated [Table 66]. This did not represent a statistically significant difference compared to tumours in older patients (P = 0.1482).

Table 66: Comparison of tumour differentiation by age group

| | ≤ 40 years old | > 40 years old |
|---|---|---|
| Poorly differentiated | 10 cases | 59 cases |
| Well or moderately differentiated | 1 case | 25 cases |
| Chi squared test | P = 0.1482 | |

The aforementioned eleven cases were comprised of three pT1 tumours (27%), two pT2 tumours (18%), five pT3 tumours (45%) and one pT4 (10%). No significant difference (P = 0.5106) was present when comparing depth of tumour invasion between the ≤ 40 and >40 year age groups [Table 67].

Table 67: Comparison of tumour depth amongst age groups:

| | ≤ 40 years old | > 40 years old |
|---|---|---|
| pT1 & pT2 | 5 cases | 47 cases |
| pT3 & pT4 | 6 cases | 37 cases |
| Chi squared test | P = 0.5106 | |

Six tumours (55%) arose in a proximal location while 5 tumours (45%) were distally located. No significant difference (P = 0.9296) was present when comparing tumour location between the younger and older groups [Table 68].

Table 68: Comparison of tumour location amongst age groups:

| | ≤ 40 years old | > 40 years old |
|---|---|---|
| Proximal tumours | 6 cases | 47 cases |
| Distal tumours | 5 cases | 37 cases |
| Chi squared test | P = 0.9296 | |

Seven of the eleven cases (64%) demonstrated aberrant E-cadherin (extracellular domain) immunolocalisation. This was significantly greater (P = 0.025) compared to aberrant immunolocalisation in tumours in patients over the age of 40 years [Table 69].

Table 69: Comparison of E-cadherin (extracellular domain) immunolocalisation between age groups.

| | ≤ 40 years old | > 40 years old |
|---|---|---|
| Aberrant E-cadherin immunolocalisation | 7 cases | 25 cases |
| Normal E-cadherin localisation | 4 cases | 59 cases |
| Chi squared test | P = 0.025 | |

No significant differences (P = 0.933) in aberrant E-cadherin (cytoplasmic domain), β-catenin (P = 0.732), Her2/neu (P = 0.789) or Dishevelled (P = 1.000) immunolocalisation were found between the two age groups.

# 4. Discussion

## 4.1 Age

The age range for all cases was 18 – 84 years, with a mean age of 57.1 years. This is similar to the findings described in a cohort of 34 cases of gastric carcinoma in a South African study. [7] These findings confirm that gastric adenocarcinoma is a disease of the middle aged and elderly.

Eleven cases were identified in patients 40 years or younger, suggesting that tumours in patients under the age of 40 years are uncommon. However, a higher proportion of young gastric adenocarcinoma cases (11%) were noted in our study cohort, when compared to the 5-6% described in the literature. [8, 9, 102] Although not a statistically significant finding, tumours occurring in patients 40 years and younger showed a 0.83 : 1 male to female ratio. This finding is similar to the 0.9 : 1 male to female ratio described in the literature for this age group. [8,9]

## 4.2 Gender

There were more males (53%) than females (47%) in the study cohort, with a male to female ratio of 1.1 : 1. This finding differs markedly from the 2:1 male to female ratio described in the Surveillance, Epidemiology and End Results (SEERs) database [102]. The exact reason for the increased proportion of female patients is unclear, though a possible reason is the small number of cases in our study. The study numbers were also probably too small to achieve a statistically significant difference in gender amongst the gastric adenocarcinoma subgroups.

## 4.3 Tumour type, location and differentiation

A predominance of diffuse type adenocarcinomas occurs in epidemiologically low risk regions while intestinal type tumours are more common in high risk areas. [6] South Africa is considered a low-to-intermediate risk region for the development of gastric carcinoma. [3] Tumours occurring in low risk areas show a proximal distribution. [103]. Fifty three percent (53%) of cases within our study showed diffuse type morphology and 57% had a proximal distribution. Although no statistically significant differences between tumour location and tumour subtype were apparent, the above findings confirm that our cohort is representative of tumours found within in the general population.

Corroboratively, 72% of our cases were poorly differentiated, with a significantly higher proportion of diffuse type adenocarcinomas being poorly differentiated compared to intestinal type adenocarcinomas.

Ninety percent (90%) of tumours occurring in patients 40 years and younger were poorly differentiated, confirming the findings described in a previous Western Cape cohort [8] and within the broader literature [9, 105]

## 4.4 Her2/Neu

Statistically significant differences in immunohistochemically detected Her2/neu overexpression were noted between the tumour subgroups. Twelve percent (12%) of the total cases demonstrated overexpression, which included a significantly greater proportion (23%) of intestinal-type tumours compared to only 3 of 31 cases of diffuse-type tumours which showed overexpression.

These findings correlate well with previous reports in the literature, confirming the morphologic-immunophenotypic association of Her2/neu overexpression and intestinal-type morphology. [85-87] In addition, the findings are similar to the reported proportion (15-25%) of gastric carcinomas which overexpress Her2/neu. [84-87].

Targeted therapy (Trastuzumab) is currently approved for the treatment of advanced gastric adenocarcinoma and metastatic gastric adenocarcinoma, if the carcinoma demonstrates unequivocal evidence of Her2/neu overexpression (by immunohistochemistry) or amplification (by FISH). Knowledge of the Her2/neu status thus plays an integral role in patient management by identifying patients who may benefit from molecular targeted therapy.

## 4.5 E-cadherin

A significantly higher proportion of diffuse-type tumours showed abnormal E-cadherin (extracellular domain) immunolocalisation compared to intestinal type tumours. This is similar to the reported findings [81, 82, 83, 90, 105] of diffuse-type tumours showing abnormal immunolocalisation.

Five percent, twelve percent and zero percent of intestinal, diffuse and mixed tumours respectively, showed abnormal E-cadherin (cytoplasmic domain) immunolocalisation. This did not represent a statistically significant difference.

However, a statistically significant correlation was noted in diffuse type carcinomas showing abnormal immunohistochemical E-cadherin localisation for both cytoplasmic and extracellular clones.

When a comparison of tumours occurring in the younger age group (40 years and younger) was made, 7 of the 11 cases showed aberrant E-cadherin (extracellular domain) immunolocalisation. This was significant when compared to aberrant immunolocalisation in tumours in patients over the age of 40 years. This confirms the findings described within the broader literature [8, 9, 105], once again highlighting that early-onset gastric carcinomas show abnormalities in E-cadherin protein expression.

An interesting point regarding the exact mechanism for the aberrant immunohistochemical expression is raised, as aberrant E-cadherin localisation may be due to a mutation (germline or somatic) in the *CDH1* gene itself or epigenetic alterations of *CDH1* promoter regions. Further molecular investigation of our cohort would provide valuable answers.

## 4.6 WNT pathway

Although only 3 of the total 97 cases showed immunohistochemically detected abnormalities in both E-cadherin (cytoplasmic domain) and β-Catenin, all of these 3 cases occurred in diffuse-type tumours. This represented a statistically significant correlation between abnormal E-cadherin (cytoplasmic domain) immunolocalisation and abnormal β-Catenin immunolocalisation amongst diffuse-type tumours.

These findings support the argument for the integrally linked role of E-cadherin and β-catenin within the WNT signalling pathway. When E-cadherin expression is downregulated, the loss of E-cadherin immunohistochemical expression results in release of β-catenin into the cytoplasm and subsequent translocation to the nucleus. [68, 70, 94]

In contrast, 8 of the 97 cases showed abnormalities in both E-cadherin (extracellular domain) and β-Catenin. However, no statistically significant correlations between abnormal E-cadherin (extracellular domain) immunolocalisation and abnormal β-Catenin immunolocalisation were found in the combined group of gastric adenocarcinomas as a whole, or within the constituent tumour subtypes (viz. intestinal, diffuse and mixed).

Fourteen of the 97 (14%) cases showed abnormal immunolocalisation of β-catenin, with aberrant immunolocalisation noted within all tumour subgroups (13%, 16% and 14% of intestinal type, diffuse type and mixed type tumours, respectively). No significant differences were evident between the subtypes of gastric adenocarcinoma. This proportion is lower than the 27% detected immunohistochemically in a cohort of 303 cases by Woo et al [99], who also showed that 37% of diffuse type tumours demonstrated altered β-catenin staining.

While Woo et al, defined altered β-catenin staining as “strong nuclear staining in more than 10% of cancer cells or loss of membranous expression, through either no immunoreactivity at the membrane or less than 10% of the tumor cells with positive membranous staining,” our study used the protocol as defined by Jass et al, 2003. [92]

The use of a different protocol in the interpretation of staining and our small study numbers are felt to contribute both to the statistical insignificance and the contrasting results when compared to the literature.

Whilst the literature shows a strong association between nuclear β-catenin accumulation and intestinal-type morphology [101], this was not apparent in our cohort of cases. In contrast, more diffuse type (16%) than intestinal type (13%) tumours showed aberrant β-catenin immunolocalisation. The exact reason for this discrepancy is not known. The underlying molecular mechanism for nuclear β-catenin accumulation may have played a role, as mutations in the *β-catenin* gene itself, WNT signalling pathway activation or *APC* gene mutations could all result in nuclear β-catenin accumulation.

The direct role of Dishevelled in gastric carcinoma pathogenesis is not well established. This has proved to be the case within our study cohort as well, with all 97 cases demonstrating moderate to intense cytoplasmic staining in more than 5% of tumour cells. No statistically significant difference in Dishevelled immunostaining was evident between the tumour subgroups. Further investigation is required to elucidate the role of Dishevelled in gastric carcinoma.

## 4.7 Study design

Our study was limited to gastric resection specimens rather than biopsy samples in an attempt to overcome the problem of tumour heterogeneity. Differing morphology and immunohistochemical expression profiles may be present within the same tumour. With only a small biopsy specimen, the tumour present in the biopsy may not be representative of the overall tumour morphology. This has an impact on tumour typing and grading. Larger resection specimens may highlight focal areas of immunohistochemical staining that may not have been detected on a limited biopsy sample.

A challenge in this study was finding adequate sample numbers. Our study cohort was limited to cases starting from 2003, as our current laboratory information system (DISA) was implemented in January 2003. Obtaining information from older databases proved very challenging, precluding cases prior to 2003 from being included in the study cohort.

A limitation of this study was inadequate patient follow-up to allow comparison of prognosis and survival rates between the tumour subgroups, and comparison with their immunohistochemical profiles.

Pre-analytical variables such as type of tissue fixation, concentration of tissue fixative, duration of tissue fixation and preparation of the gross sample represented factors that could not be controlled directly within this study. It is known that under-fixation and the use of tissue preservatives other than formaldehyde, can impact on antigen retrieval and result in failure of immunohistochemical staining. [106]

A standard staining method was employed for all cases and each primary antibody was optimised prior to starting the study. Satisfactory positive and negative external controls were used for each antibody. In addition, positive internal controls were noted.

In an attempt to avoid bias, all slides were coded, thus preventing the observer from knowing to which tumour subtype the case belonged. An attempt to limit intra-observer variability was made, whereby all cases were scored by the same

pathologist, using the same microscope in as few sittings as possible. Scoring of the cases was checked by the supervising pathologist.

It is hoped that the use of defined grading systems for each antibody, meticulous attention to cellular location of immunohistochemical staining and the use of polymer-based immunohistochemical kits, minimised false-positive results within our study.

A strength of our study is the division of tumours into intestinal, diffuse and mixed types, so that the effect of age variables and immunohistochemical staining can be factored into the comparison between the tumour groups.

# 5. Conclusion and recommendations

Gastric adenocarcinoma is predominantly a disease of the middle aged and elderly within the Western Cape, confirming the findings reported in the literature.

While gastric adenocarcinoma is uncommon in patients under the age of 40 years, an increased proportion of cases within the Western Cape occur in those 40 years and younger compared to other reported populations.

When gastric adenocarcinomas do occur in patients under the age of 40 years, they are invariably poorly differentiated, confirming the reported literature findings. In addition, gastric adenocarcinomas in young patients show a significantly higher proportion of abnormal E-cadherin immunolocalisation versus tumours in older patients.

Despite gastric carcinoma worldwide occurring in twice as many males as females, our cohort of patients showed almost equal gender distribution.

As reported in the literature, Her2/neu overexpression is significantly more frequent in tumours with intestinal-type morphology compared to those with diffuse-type morphology.

Tumours with diffuse-type morphology show aberrations in E-cadherin immunolocalisation. When aberrant immunolocalisation does occur, it involves either the E-cadherin extracellular domain alone or results from combined defects in the cytoplasmic and extracellular domains.

As reported, E-cadherin and β-catenin are integrally linked within the WNT signalling pathway. Aberrations in the normal functioning of E-cadherin result in abnormal accumulation and aberrant localisation of β-catenin. Tumours with combined defects in E-cadherin and β-catenin demonstrate diffuse-type morphology.

# 6. Appendix

Appendix 1: Summary of cases of gastric adenocarcinoma

| Case number | Age (years) | Gender | Tumour type |
|---|---|---|---|
| 1 | 78 | Male | Intestinal |
| 2 | 58 | Female | Diffuse |
| 3 | 54 | Male | Intestinal |
| 4 | 18 | Female | Mixed |
| 5 | 53 | Male | Diffuse |
| 6 | 40 | Male | Diffuse |
| 7 | 38 | Female | Diffuse |
| 8 | 77 | Female | Intestinal |
| 9 | 60 | Male | Intestinal |
| 10 | 54 | Male | Mixed |
| 11 | 51 | Female | Mixed |
| 12 | 84 | Male | Intestinal |
| 13 | 74 | Female | Diffuse |
| 14 | 60 | Male | Intestinal |
| 15 | 59 | Female | Intestinal |
| 16 | 59 | Male | Intestinal |
| 17 | 69 | Male | Intestinal |
| 18 | 50 | Female | Intestinal |
| 19 | 44 | Male | Diffuse |
| 20 | 64 | Female | Intestinal |
| 21 | 53 | Male | Intestinal |
| 22 | 43 | Female | Mixed |
| 23 | 78 | Male | Intestinal |
| 24 | 36 | Male | Diffuse |
| 25 | 79 | Male | Intestinal |

| | | | |
|---|---|---|---|
| 26 | 48 | Male | Diffuse |
| 27 | 70 | Female | Intestinal |
| 28 | 43 | Female | Diffuse |
| 29 | 46 | Male | Mixed |
| 30 | 47 | Female | Diffuse |
| 31 | 52 | Male | Intestinal |
| 32 | 49 | Male | Intestinal |
| 33 | 41 | Female | Intestinal |
| 34 | na | Female | Intestinal |
| 35 | 60 | Male | Intestinal |
| 36 | 70 | Female | Intestinal |
| 37 | 72 | Female | Intestinal |
| 38 | 46 | Male | Diffuse |
| 39 | 54 | Female | Intestinal |
| 40 | 72 | Female | Intestinal |
| 41 | 81 | Male | Intestinal |
| 42 | na | Female | Intestinal |
| 43 | 82 | Male | Intestinal |
| 44 | 36 | Male | Diffuse |
| 45 | 46 | Female | Mixed |
| 46 | 66 | Male | Intestinal |
| 47 | 43 | Male | Intestinal |
| 48 | 72 | Male | Intestinal |
| 49 | 62 | Male | Intestinal |
| 50 | 64 | Male | Intestinal |
| 51 | 69 | Male | Intestinal |
| 52 | 73 | Female | Intestinal |
| 53 | 52 | Male | Intestinal |
| 54 | 57 | Male | Mixed |
| 55 | 61 | Male | Diffuse |
| 56 | 76 | Female | Intestinal |
| 57 | 73 | Female | Diffuse |

| | | | |
|---|---|---|---|
| 58 | 57 | Male | Intestinal |
| 59 | 54 | Female | Diffuse |
| 60 | 58 | Female | Diffuse |
| 61 | 53 | Female | Diffuse |
| 62 | 42 | Male | Intestinal |
| 63 | 52 | Female | Diffuse |
| 64 | 36 | Female | Diffuse |
| 65 | 33 | Female | Diffuse |
| 66 | 78 | Female | Diffuse |
| 67 | 60 | Male | Diffuse |
| 68 | 37 | Female | Diffuse |
| 69 | 62 | Female | Diffuse |
| 70 | 36 | Male | Diffuse |
| 71 | 57 | Female | Diffuse |
| 72 | 41 | Female | Diffuse |
| 73 | 62 | Female | Diffuse |
| 74 | 73 | Female | Diffuse |
| 75 | 64 | Male | Diffuse |
| 76 | 69 | Female | Diffuse |
| 77 | 64 | Male | Diffuse |
| 78 | 32 | Female | Intestinal |
| 79 | 60 | Female | Diffuse |
| 80 | 55 | Male | Diffuse |
| 81 | 65 | Female | Diffuse |
| 82 | 44 | Female | Diffuse |
| 83 | 75 | Female | Diffuse |
| 84 | 69 | Female | Diffuse |
| 85 | 55 | Male | Diffuse |
| 86 | 61 | Female | Diffuse |
| 87 | 60 | Male | Diffuse |
| 88 | 67 | Male | Diffuse |
| 89 | 40 | Male | Diffuse |

| | | | |
|---|---|---|---|
| 90 | 42 | Female | Diffuse |
| 91 | 57 | Male | Diffuse |
| 92 | 44 | Male | Diffuse |
| 93 | 82 | Male | Diffuse |
| 94 | 49 | Female | Diffuse |
| 95 | 56 | Male | Diffuse |
| 96 | 58 | Female | Diffuse |
| 97 | 54 | Male | Diffuse |

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