Investigating the Relationship between miRNA expression and Epithelial Mesenchymal Transition in colorectal cancer.

By

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Declaration

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Dedication

I dedicate this dissertation to my parents (Mr Mziwandile Leslie and Mrs Noludwe Veronica Jaca) and siblings (Nonqaba and Sinoyolo) in honour of their love and support.

Acknowledgements

First and foremost, I would like to extend my appreciation to my supervisor A/Professor Richard Naidoo for his supervision and support. He is an amazing teacher. I appreciate everything he has done for me throughout this journey. My co-supervisor, Dr Michael Locketz has played a tremendous role in making sure that the pathology aspect of this investigation was carried out successfully. I would also like to express my gratitude to all the students and staff members at the Division of Anatomical Pathology: Mrs Subash Govender for helping with the sectioning in the histology laboratory. I also appreciate Professor Dhiren Governder's insightful comments during research meetings, they were extremely helpful. Mrs Pumza Magangane assisted me with my statistical analysis, I really thank her for that. I extend my gratitude to Dr Luiz Zerbini for allowing me to conduct all miRNA experiments in the International Centre for Genetic Engineering and Biotechnology (ICGEB) laboratory. Mr Henri Carrara from Public Health, University of Cape Town, played an amazing role with the statistical analysis, his help is much appreciated.

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Abstract

Title: Investigating the relationship between miRNA expression and epithelial mesenchymal transition (EMT) in colorectal cancer.

Introduction: Epithelial-mesenchymal transition (EMT) is characterized by the loss of an epithelial phenotype and gain of a mesenchymal phenotype, i.e., migratory and metastatic properties. The EMT process is therefore characterized by a low expression of E-cadherin and high expression of mesenchymal markers (e.g., N-cadherin, snail and vimentin). It is stated that cells which have undergone EMT also gain stem cell features. Therefore, both EMT and stem cell phenotypes have been implicated in carcinogenesis and metastasis of tumour cells. Furthermore, EMT is regulated by small non-coding molecules (miRNAs) that either function as tumour suppressors or oncogenes (oncomirs). Tumour suppressor miRNAs reverse EMT while oncomirs activate it. Therefore, investigating the relationship between miRNAs and EMT is important in addressing metastasis of colorectal cancers (CRC).

Aims and Objectives: The aim of the study was to determine the association between miRNA (miRNA-21 and miRNA-34a) expression levels and EMT in CRC. In addition, this investigation aimed to correlate miRNA and EMT data with clinicopathologic features of the study cohort.

Methodology: A total of 100 CRC (including 8 known HNPCC cases) Formalin Fixed Paraffin Embedded (FFPE) tissue blocks and their corresponding H&E slides were collected from the archives of the Division of Anatomical Pathology at the University of Cape Town. Subsequently, the FFPE tissue blocks were sectioned at 3µm and IHC analysis of 4 EMT markers (E-cadherin, N-cadherin, snail-1 and vimentin) and 1 stem cell marker (CD44V6) was performed. The stains were then evaluated and scored by a pathologist. The IHC data were then correlated with clinicopathologic features. Furthermore, 59 cases (FFPE tissues and corresponding H&E slides) which included the 8 HNPCCs were randomly selected for miRNA analysis. The H&Es were examined by a pathologist to demarcate normal and tumour regions. RNA was then extracted from 59 tumours and 12 normal tissues using a High Pure FFPET Isolation Kit (Roche). Subsequently, cDNA was synthesized and qRT-PCR was performed to determine the expression levels of miRNA-21 and miRNA-34a. MiRNA-21 and miRNA-34a expression levels were ascertained using the relative quantification method. Moreover, the clinical significance of the two miRNAs was evaluated in relation to MSI status. Therefore, IHC analysis of MLH1, MSH2 and MSH6 mismatch repair proteins was performed on the Ventana platform. Statistical analysis was performed using Fisher's and Pearson's Chi Square

tests in Stata 12 to correlate EMT and clinicopathologic data. Additionally, the Mann-Whitney non-parametric test in GraphPad prism 6 was used to determine miRNA-21 and miRNA-34a expression in relation to EMT and MSI data.

Results: Our results showed low expression of E-cadherin in 77% of cases. In addition, there was decreased expression of N-cadherin and vimentin in 98% whilst snail-1 expression was decreased in 65% of the cases. Low expression of CD44v6 was also seen in 78% of the cases. There was no correlation between EMT/stem cell markers and clinicopathologic data. Furthermore, increased miRNA-21 expression was significantly associated with grade, lymph node metastasis and age of patients. There was a significant correlation between high miRNA-21 expression and down-regulated snail-1 and N-cadherin expression. MiRNA-34a expression was not associated with any of the clinicopathologic features. In addition, high miRNA-34a expression was linked with low expression of snail-1 and CD44v6. Increased miRNA-21 expression was related with MSS tumours, whereas there was no relationship between miRNA-34a and MSI status.

Conclusion: Our investigation shows that there is an inverse association between miRNA (miRNA-21 and miRNA-34a) expression and two EMT (N-cadherin and snail-1) markers in our colorectal cancer cohort. Our data also show that both miRNA-21 and miRNA-34a cannot be used as biomarkers to determine progression of the cancer. Contrary to previous studies, our findings indicate that miRNA-21 does not activate EMT in this CRC cohort. However, similar to other studies our results confirm that miRNA-34a may be repressing snail-1 expression, thereby inhibiting EMT in the cancer.

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List of Abbreviations

β-TrCP – Beta-Transducin Repeat-Containing Protein

3′ UTR – Three prime Untranslated Region

5FU – Fluorouracil

ALDH1A1 - Aldehyde Dehydrogenase 1 family member A1

APC - Adenomatous Polyposis Coli

ATF- Associated-transcription factors

B-RAF - B-Raf Proto-oncogene

bHLH - Basic Helix-Loop-Helix

Bmi-1- BMI1 proto-oncogene, polycomb ring finger

BRG1 – Brahma-related gene-1

c-MET – c- Met Proto-Oncogene Tyrosine Kinase

c-MYC - Avian myelocytomatosis virus oncogene cellular homolog

CBCs - Crypt Base Columnar Cells

CD133 - Cluster of Differentiation 133

CD44v6 - Cluster of Differentiation 44 variant 6

Cdc25a - Cell division cycle 25 homolog A

CDC4 - cell division control protein 4

CDH1 - Cadherin 1, type 1

cDNA - Complementary DNA

CDK - cyclin-dependent kinases

CK22 - Cytokeratin 22

CIN - Chromosomal instability

CRC - Colorectal Cancer

CSC – Cancer Stem Cells

CSL - C protein binding factor I/suppressor of hairless/lag-1

Ct – Threshold Cycle

CTNNB1 - Catenin (cadherin-associated protein), beta 1

DAB - Diaminobenzidine

Dclk1 - Doublecortin-like kinase 1

dH_2O - Distilled water

DLL - Delta-like

DBD - DNA-binding domain

DNA - Deoxyribonucleic Acid

DNase - Deoxyribonuclease

dNTP - Deoxynucleoside triphosphate

E-cadherin - Epithelial-cadherin

EDTA - Ethylenediamine Tetraacetic Acid

EMC - Extracellular Matrix

EMT - Epithelial Mesenchymal Transition

ERK - Extracellular mitogen-activated kinases

FAP - Familial Adenomatous Polyposis

FFPE - Formalin Fixed Paraffin Embedded

FGF - Fibroblast Growth Factor

FGFR1 - Fibroblast growth factor receptor 1

FOS – FOS oncogene

Fz - Frizzled

GSK-3ß - Glycogen Synthase Kinase-3Beta

H_2O_2 - Hydrogen peroxide

H&E - Haematoxylin and Eosin

HDAC1 - Histone Deacetylase

hMLH1 - Human Multi Homolog 1

HNPCC - Hereditary nonpolyposis colorectal cancer

HoxA7 - Homeobox A7

HPs - Hyperplastic Polyps

HREC - Human Research Ethics Committee

ICN - Intracellular

ITF-2 - Immunoglobulin Transcription Factor 2

IF - Intermediate Filament

IHC – Immunohistochemistry

JUN - Jun Proto-Oncogene

K-RAS - Kirsten Rat Sarcoma Viral oncogene

LEF - Lymphoid Enhancer Factor

LRCs - Label-Retaining Cells

Lgr5 - Leucine-rich repeat-containing G-protein coupled receptor 5

LRP - Lipoprotein-related

MET - Mesenchymal Epithelial Transition

$MgCl_2$ - Magnesium Chloride

miRNA - Micro RNA

$MnCl_2$ - Manganese (II) chloride

MOS – Moloney murine sarcoma oncogene

MRE11A - MRE11 homolog A, double strand break repair nuclease

MSS - Microsatellite Stable

MTA 1 - Metastasis Associated Protein 1

MLH1 - Multi Homolog 1

MMP-7 - Matrix Metalloproteinase-7

MMR - Mismatch Repair

MPs - Mixed Polyps

mRNA - Messenger RNA

MSI - Microsatellite Instability

MSH2 - Muscle Segment Homeodomain Protein2

MSH3 - Muscle Segment Homeodomain Protein3

MSH6 - Muscle Segment Homeodomain Protein6

MSH-H – High Microsatellite Instability

MSI-L – Low Microsatellite Instability

MSS – Microsatellite Stable

N-cadherin - Neuronal-cadherin

NCI - National Cancer Institute

NF-κB - Nuclear Factor-kappa B

NF-1 – Neurofibromatosis Type 1

NFIB - Nuclear factor 1 B-type

NGS - Normal Goat Serum

p21 - Protein 21

PI3-K - Phosphoinositide 3-kinase

PBS - Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDCD4 - Programmed Cell Death 4

PMS1 - Postmeiotic Segregation Increased-1

PMS2 - Postmeiotic Segregation Increased-2

PSSP - Power and Sample Size Program

PTEN - Phosphatase and Tensin Homolog

Pre-miRNA - Precursor miRNA

RAF – RAF kinase

Rb - Retinoblastoma

R-SMAD - Receptor modulated SMAD

RECK - Reversion-inducing-cysteine-rich protein with kazal motifs

RHOB - Ras homolog gene family, member B

RNA- Ribonucleic Acid

RNase - Ribonuclease

RT-PCR - Reverse Transcriptase-Polymerase Chain Reaction

SDS - Sodium Dodecyle Sulfate

SIRT1 - silent mating type information regulation 2 homolog

SMA - Smooth Muscle Actin

SMAD4 – SMAD family member 4

SPRY2 - Sprouty 2

SRC - Proto-oncogene tyrosine-protein kinase Src

SSA - Sessile Serrated Adenoma

TCF - T-cell Specific Factor

TGF-β - Transforming Growth Factor-Beta

TIMP3 – TIMP metallopeptidase inhibitor

TIAM1 - T-lymphoma invasion and metastasis-inducing protein 1

TMEM49 - Transmembrane protein 49

TP53 - Tumour Protein p53

TPM1 - Tropomyosin 1

TSA - Traditional Serrated Adenoma

Zeb - Zinc finger E-box-Binding homeobox protein

V600E - valine (V) to a glutamic acid (E), position 600

VEGF - Vascular Endothelial Growth Factor

xg – Times gravity

ZFs - Zinc Fingers

Chapter One

1. Background

1.1. Colorectal cancer incidence

Colorectal cancer (CRC) is rated as the fourth most frequent malignancy in the world (Globocan, 2012). It is the second most frequent cancer in women and the third in men. Incidence and death rates are less frequent in developed (e.g., Central and Eastern Europe, North America and Eastern Asia) than developing countries (e.g., Central America). CRC is also common in all the different parts of Africa: Southern Africa, Northern Africa, Western Africa, Middle Africa and Eastern Africa. In South Africa, the estimated incidence rates are about 6.1%. Moreover, there are high death rates in the less developed countries, reflecting a poor survival of the disease (Globocan, 2012).

1.2. Colorectal cancer subtypes

Colorectal cancer (CRC) develops as a result of the abnormal growth of cells lining the mucosa of the colon and rectum. It is classified into sporadic and hereditary (e.g., non-polyposis colorectal cancer) types (Martinez-Urena et al., 2013). Sporadic CRC is the most frequent type which is commonly seen in older people, especially males. Furthermore, it is seen in the distal part of the colon and is often poorly differentiated (Nakanishi et al., 2013). The Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant disorder that accounts for about 3% to 5% of the disease (Diergaarde et al., 2007). HNPCC is characterized by high penetrance, early-onset and an increased risk of certain extra-colonic cancers, including cancers of the endometrium, stomach, small bowel, ovary, hepatobiliary tract, renal pelvis, and ureter (Lynch et al., 1996). This type is commonly seen in the proximal colon. The pathogenesis of sporadic and hereditary types of CRC is associated with environmental and genetic risk factors.

1.3. Risk factors

Several risk factors are attributed to the development of colorectal cancer and these involve environmental, genetic and epigenetic factors (changes in biological pathways).

1.3.1. Environmental risk factors

Various environmental factors are implicated in carcinogenesis and these include dietary factors, poor nutrition and tobacco smoking.

1.3.1.1. Dietary factors and poor nutrition

An imbalanced diet has also been identified as a common factor that is linked with the development of colorectal cancer. A diet lacking in fresh fruit and vegetables as well as dairy products is usually deficient in vitamins A, C and E (Mahfouz et al., 2014). The lower intake of these nutrients may cause the colorectal mucosa to be more predisposed to carcinogens. Furthermore, the absence of trace elements such as zinc, selenium, molybdenum, silicon, nickel, iron, iodine, phosphorus, potassium and magnesium from the diet have also been reported as risk factors (Song et al., 2015). Moreover, a high intake of nitrosamines and nitrosamine precursors (nitrates, nitrites, secondary and tertiary amines) are also major risk factors (Santarelli et al., 2008).

1.3.1.2. Tobacco Smoking

Both smoking and excessive alcohol consumption are also considered as important risk factors for the disease (Klarich et al., 2015). It has been hypothesised that smoking together with alcohol (which acts as a solvent) is detrimental to the development of the disease (Gao et al., 2013). Furthermore, tobacco is considered as carcinogenic when used in any form: e.g., cigarettes, pipe tobacco, cigar, snuff and chewing tobacco. However, pipe and hand-rolled cigarette smoking are more carcinogenic than commercial cigarette smoking (Lindsay et al., 2009).

1.3.2. Genetic factors

Changes or mutations in oncogenes, tumour suppressor, DNA repair and cell cycle genes may predispose individuals to developing cancer.

1.3.2.1. Oncogenes

Previous investigations indicated that cells could be transformed in culture by DNA viruses and retroviruses. Genes having a similar sequence to those of the viruses were seen in the DNA of normal cells (Freeman, 2000). Those genes regulate cell growth and differentiation; however when they are abnormally activated, they can lead to the development of cancer. The normal cellular genes are called proto-oncogenes and their activated forms are named oncogenes (Cooper, 2000). Oncogenes contribute to the development of cancer by promoting cell proliferation or reducing apoptosis. In cancers, proto-oncogenes are activated by genetic mutations, namely, point mutations, gene amplifications and chromosomal abnormalities (Pino et al., 2010). A single mutation in one of the two gene copies is sufficient to promote abnormal growth, therefore, oncogenes are said to be dominant (Freeman, 2000). Several oncogenes have been reported to contribute to the initiation of cancer and these include growth factors (*B-raf*,

K-ras, *raf*, *mos*, *src*), transcriptional factors (*c-myc*, *jun*, *fos*), and cell-cycle genes (*cyclin D1*, *CDK4*), (Freeman, 2000).

1.3.2.2. Tumour Suppressor Genes

Unlike oncogenes, tumour suppressor genes are negative modulators of cell proliferation. Tumour suppressor genes function to hinder cell proliferation by inducing apoptosis. However, when these genes are mutated, their function is lost, consequently leading to the formation of cancer (Yuspa et al., 1994). Knudson (1971) showed that retinoblastoma (Rb), which is a childhood tumour, is a result of an inactivation in both copies of the *Rb* gene (Figure 1.1.). This is referred to as the "two hit" hypothesis (Knudson, 1971).

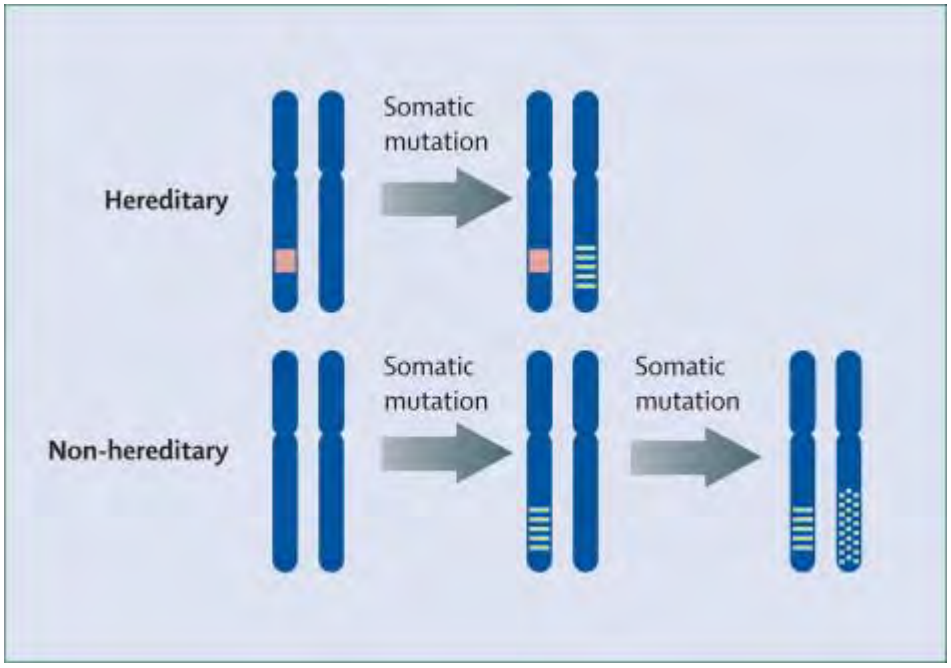


Figure 1. 1: The "two hit" hypothesis model showing that in hereditary retinoblastoma, the first hit is an inherited mutation while the second hit is a somatic mutation of the second allele. In the case of non-hereditary tumours, two somatic mutations affect both alleles of the *Rb* gene (Levine, 1995).

Furthermore, tumour suppressor genes are classified into three subgroups of function i.e., gatekeepers, care takers and landscapers (Deininger, 1999; Srivastava et al., 2010). Gatekeepers are genes that impede cell growth or stimulate differentiation and apoptosis, e.g., *APC*, *Rb*, *p53* and *NF-1* (Ligius, 1993; Gnarra et al., 1994). Germline mutation of a gatekeeper gene leads to a cancer risk of at least 100 times greater than in the general population. Caretaker genes are involved in DNA repair and maintenance of genomic integrity (Eng et al., 2003). Lastly, landscapers are the genes (e.g., *TSP-1, NF-1, RB, PTEN and DPC4*) which stimulate cancer development through tissue dysplasia. These genes modulate tissue morphogenesis, intercellular signaling and differentiation (Nigro et al., 1989; Wales et al., 1995; Macleod, 2000).

1.3.2.3. DNA repair genes

DNA repair is a mechanism by which a cell identifies and corrects errors e.g., repeat sequences, mismatches and strand breaks which may occur during DNA replication (Wang, 2015). Several DNA repair pathways are involved in repairing damaged DNA. These include mismatch repair (MMR), base-excision repair (BER), nucleotide-excision repair (NER), translesion synthesis (TLS), homologous recombination (HR), non-homologous end joining (NHEJ), Fanconi anemia (FA) and O6-methylguanine DNA methyltransferase (MGMT) pathways (Dietlein et al., 2014). The most common pathways associated with DNA repair in cancer are the BER, NER and MMR. The damage is removed and the DNA sequence is repaired by a DNA polymerase in all three pathways. The BER involves enzymes called DNA glycosylases which identify a specific type of altered base in DNA and therefore remove it (Alberts et al., 2002). Furthermore, the NER system repairs damages in the structure of the DNA double helix.

MMR recognizes incorrect insertions, deletions and mis-incorporations of bases (Potenski and Klein, 2014). The incorrect number of bases is then detected by the MutSa (*MSH2/MSH6*), MutSb (*MSH2/MSH3*) and MutLa (*MLH1 and PMS2*) complexes of the MMR system. The MutSa complex identifies small mismatches whilst MutSb detects large mismatches. Additionally, the MutLa complex coordinates base mismatch repair (Perevoztchikova et al., 2013). Mutations in the DNA repair genes can lead to a failure in their repair function, which in turn allows subsequent mutations to accumulate. When these genes fail to repair DNA, irreversible DNA damage i.e., double-strand breaks and DNA cross linkages may occur. These errors can therefore lead to the initiation of cancer (Torgovnick and Schumacher, 2015).

1.3.2.4. Cell cycle genes

The cell cycle is a mechanism by which cells divide. It is divided into gap phase 0 (G0), gap phase 1 (G1), DNA synthesis (S), gap phase 2 (G2), and mitosis (M). This pathway is composed of proteins i.e. cyclins (G1/S cyclins, S cyclins, M cyclins, G1 cyclins) and cyclin-dependent kinases (CDK). These proteins form complexes and therefore modulate the progression from one stage of the cell cycle to another (Vermeulen et al., 2003). Checkpoints are identified within the cell cycle stages to ensure that events such as DNA synthesis proceed accordingly. If there is any DNA damage, the checkpoints arrest the cell cycle until the problem is repaired (Collins et al., 1997).

Molecules which regulate the G1/S phase such as proto-oncogenes and tumour suppressor genes may undergo alterations and hence contribute towards cancer formation. Additionally, checkpoints such as those responsible to check DNA damage and modulate DNA replication may also be genetically compromised (Bartek et al., 1999). A number of cell cycle genes have been implicated in carcinogenesis and these include *p53*, *cyclin D1*, *p21*, *p27*, *CDK4* and *p57* (McKay et al., 2000).

1.4. Molecular pathways underlying colorectal carcinogenesis

1.4.1. Fearon and Vogelstein model: adenoma-carcinoma sequence

In 1990, Fearon and Vogelstein introduced a model that explained the multistep process to the development of CRC. This model suggests that colorectal cancer may develop as a result of mutational activation of oncogenes and inactivation of tumour-suppressor genes. Secondly, mutations in at least four or five genes are necessary for malignant alteration. Lastly, although the genetic mutations commonly arise in a preferred order, the mutations rather than their chronologic order, is responsible for determining biologic features of the tumour (Fearon and Vogelstein, 1990). Furthermore, CRC develops as a result of the conversion of the normal epithelial mucosa from adenomas (benign tumours) to carcinomas (Figure 1.2), (Arends, 2013). This conversion is accompanied by genetic mutations of *APC, KRAS* and *TP53* genes (Yadamsuren et al 2012; Zauber et al 2013; Hershkovitz et al., 2014).

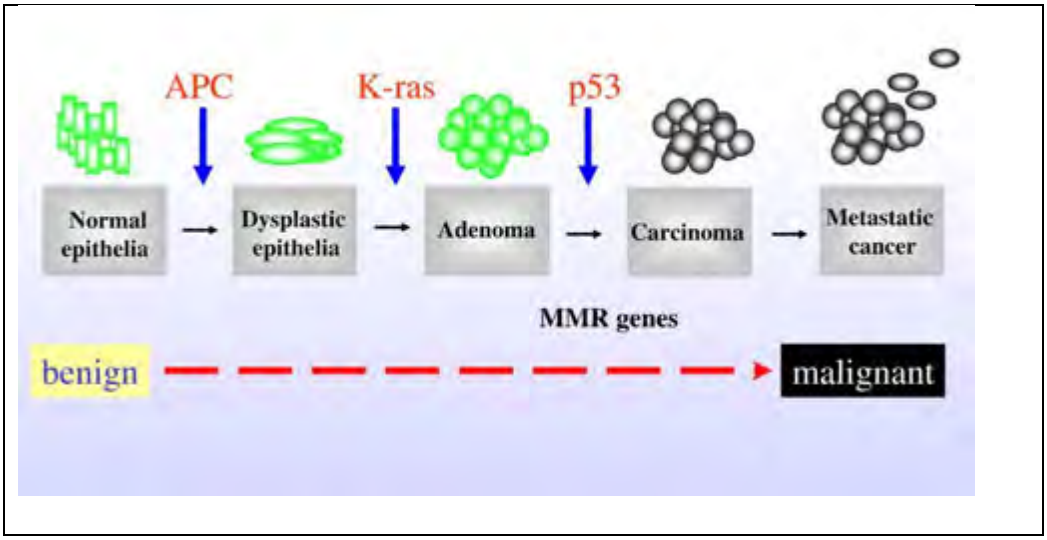


Figure 1. 2: The adenoma-carcinoma sequence involves a transition from normal epithelium to adenoma and carcinoma due to acquired molecular events e.g., mutations in three key genes (*APC*, *KRAS* and *P53*), (Smith et al., 2002).

1.4.2. Microsatellite instability

Microsatellites are short fragments of DNA dispersed throughout the genome that have repetitive sequences. Microsatellite instability (MSI) occurs as a result of alterations (decrease or increase) in microsatellites. MSI is therefore a form of genetic hyper-mutability that occurs in the genes (*MLH1, MSH2, MSH3, MSH6, PMS1* and *PMS2*) that encode DNA mismatch repair (MMR) proteins (Arends, 2013). These genetic mutations consequently result in defective MMR proteins, causing the inability to correct errors during DNA replication. In individuals with CRC, MSI is indicated by the loss of MLH1, MSH2, MSH6 and PMS2 proteins. In HNPCC patients, the predominantly affected proteins are MLHI and MSH2 due to germline mutations of *MLH1* and *MSH2* genes (Bergine et al., 2009; Sinicrope et al., 2015). Other MMR proteins are less affected. These are *MSH6* and *PMS2* caused by *MSH6* and *PMS2* germline mutations *(*Moreira et al., 2014; Karahan et al., 2015). MSI is seen in 15% of sporadic CRCs, as a result of *BRAF* V600E mutation and the silencing of *MLH1* gene through promoter hyper-methylation (Veganzones et al., 2015). Since MSI is caused by different genetic changes:

(e.g., *MLH1* hyper-methylation in sporadic cancers and *MLH1*/*MSH2* germline mutations in HNPCC), an algorithm was introduced to analyse and classify these two types of CRCs (Figure 1.3).

1.4.2.1. MSI analysis

The National Cancer Institute (NCI) proposed five microsatellite markers essential to determine MSI presence: two mononucleotides, BAT25 and BAT26, and three dinucleotide repeats, D2S123, D5S346, and D17S250 (Odenthal et al., 2009). Tumours can therefore be classified into three groups: i.e. high MSI (MSI-H) if 2 or more markers are unstable, low MSI (MSI-L) when one marker shows instability and microsatellite stable (MSS) when none of the markers show instability. In addition, immunohistochemical (IHC) analysis of the MMR proteins is a supplementary approach used to determine MSI in CRC (Figure 1.3). Knowing the MSI status in CRC patients is useful in predicting some clinical behaviour e.g., prognosis and response to treatment, hence it is important to analyse the MSI status in this disease (Parc et al., 2004; Sinicrope et al., 2011).

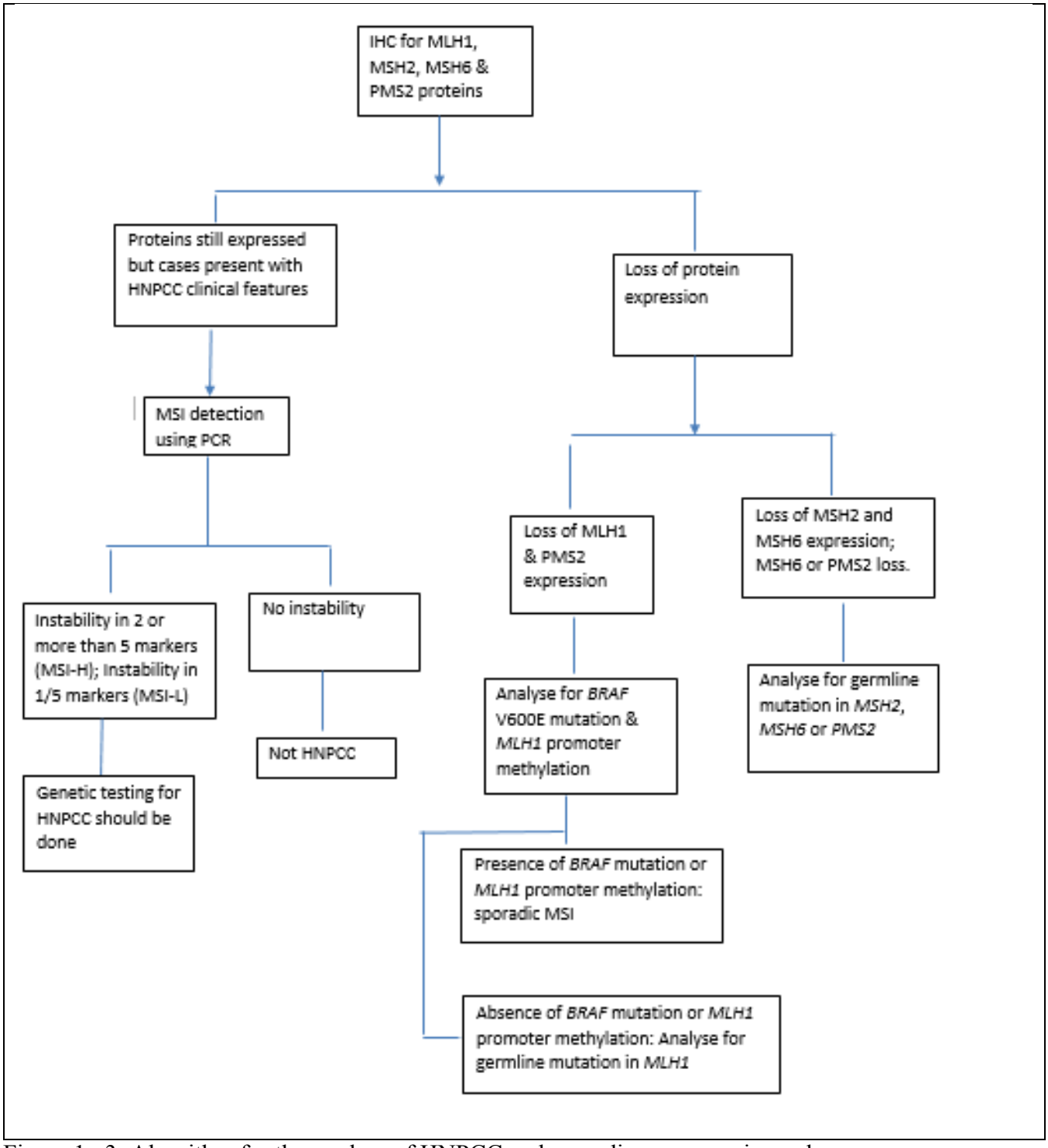


Figure 1. 3: Algorithm for the workup of HNPCC and sporadic cancers using polymerase chain reaction (PCR) and IHC tests (Geiersbach and Samowitz, 2011).

1.4.3. Chromosomal instability

Chromosomal instability (CIN) is a phenomenon where daughter cells receive an unequal number of DNA molecules and results in an unequal number of chromosomes during cell division (Orsetti et al., 2014). CIN occurs when the daughter cells do not have the same number of chromosomes as the cell they were derived from. CIN is seen in 65%–85% of sporadic colorectal cancers (Pino and Chung, 2010; Cisyk et al., 2015). It causes the loss of a wild-type copy of tumour suppressor genes, such as *CDC4*, *P53*, *MYC*, *FGFR1*, *MRE11A*, *KRAS* and *SMAD4* (Mouradov et al., 2013; Ertych et al., 2014; Beyer et al., 2015; Cisyk et al., 2015).

Furthermore, the two genetic instabilities i.e. MSI and CIN were suggested to have a joint role in the development and progression of colorectal cancer (Mouradov et al., 2013; Hveem et al., 2014). There is a direct association between MSI/CIN positive tumours and stage II/III tumours, located on the right side of the colon. However, the tumours with MSI alone were associated with a better survival than those with CIN. In addition to the above mentioned genetic instabilities, colorectal carcinogenesis may also involve modifications in certain biological pathways involving microRNA and EMT (Mouradov et al., 2013; Hveem et al., 2014).

1.5. Epigenetic factors: Changes in biological pathways

MicroRNA (miRNA) and epithelial mesenchymal transition (EMT) play a significant role in key biological pathways linked with the development of cancer. MiRNAs are known to target and bind to the promoter regions of EMT-associated transcription factors (EMT-ATFs), thereby, down-regulating or up-regulating the expression levels of these TFs. Consequently, the down-regulated or up-regulated expression of EMT-ATFs may promote CRC progression and certain clinical features e.g., invasion and metastasis of cancer cells (Pereira et al., 2015; Abba et al., 2016).

1.6. Biosynthesis of miRNAs

MiRNAs are small non-coding molecules with about 20 to 22 nucleotides. These molecules are located in the introns and occupy about 2% to 5% of the human genome (Bullock et al., 2012). They bind to their specific target messenger RNAs in the three prime un-translated (3′ UTR) region (Diaz-Lopez et al., 2014). These molecules regulate gene expression during developmental (embroyonic development), physiological (reproduction, fibrosis) and pathological (cancer development) processes (Chang and Hla, 2014). They are expressed as primary miRNAs (pri-miRNAs) in the nucleus and are processed by the RNase III enzyme (Drosha-DGCR8) to produce precursor miRNAs (pre-miRNA), (Figure 1.4). The pre-miRNA, with approximately 80 nucleotides then becomes translocated to the cytoplasm by the Exportin 5 molecule (Figure 1.4) where it becomes cleaved by RNase III enzyme (Dicer), (Figure 1.4). This results in a mature miRNA of 21 to 22 nucleotides (Chen et al., 2014).

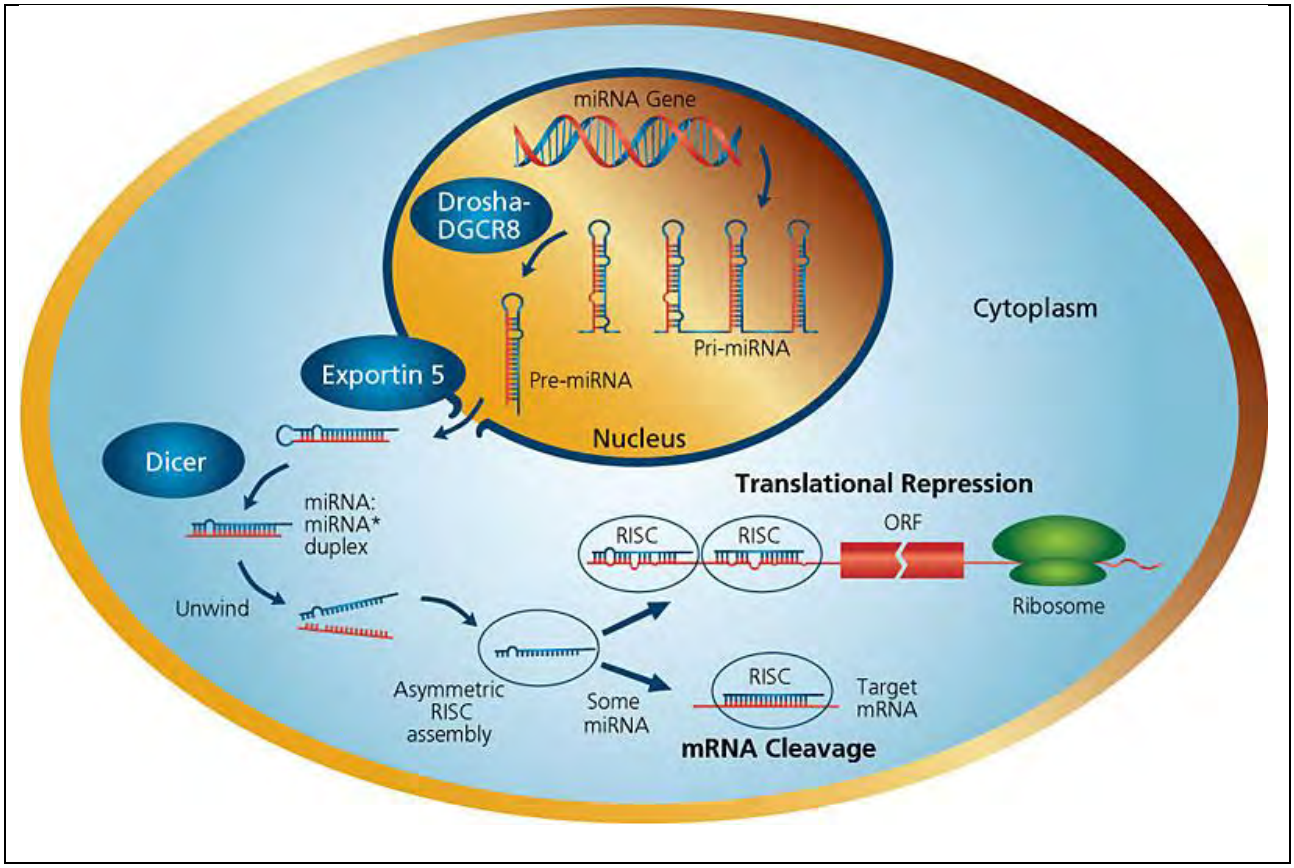


Figure 1. 4: miRNAs are processed by the RNase III enzyme (Drosha-DGCR8) in the nucleus to produce precursor miRNAs (pre-miRNA). The pre-miRNA then becomes translocated to the cytoplasm by the Exportin 5 molecule. In the cytoplasm, it is cleaved by another RNase III (Dicer) enzyme producing a mature miRNA.

1.7. Mechanisms of miRNA dysregulation in cancer

MiRNA expression is regulated through various mechanisms, namely, transcriptional factors, epigenetic changes and genetic alterations (Croce, 2009; Chen et al., 2012). These mechanisms may contribute to the abnormal expression of miRNAs in human cancers (Deng et al.., 2008).

MicroRNAs are known to directly regulate the expression of transcription factors at a post-transcriptional level. The expression of a well characterized miRNA cluster, i.e., miRNA-17 to 92a is activated by direct binding of c-MYC to its promoter region. Subsequently, these miRNAs suppress various targets, including cell cycle regulator p21 and pro-apoptotic factors, leading to increased chances of developing cancer. Similarly, miRNA-143 and miRNA-145 which are significantly down-regulated in colorectal cancer are regulated by KRAS. Tumour suppressive miRNAs such as miRNA-34, miRNA-15a and miRNA-145 may also be regulated by transcription factors e.g., p53, EGFR, cMYC, MUC1 and SOX2 (Feng et al., 2011; Concepcion et al., 2012; Cui et al., 2014).

DNA methylation and histone modification also form an important regulatory mechanism for miRNA regulation. In human cancers, miRNA promoter regions are known to be highly methylated. Down-regulation of tumour suppressor miRNAs, e.g., miRNA-34b and miRNA-34c is mainly caused by hyper-methylation, seen in about 90% of primary CRCs (Majid et al., 2012). Furthermore, genetic variations, including single nucleotide polymorphisms (SNPs), deletions and duplications also regulate miRNA expression. These variations are commonly seen in the mature sequence and 3' UTR, leading to the removal of target recognition sites of miRNAs and hence loss of function (Meola et al., 2009).

1.8. miRNAs in cancer

MiRNAs play an important role in the regulation of various metabolic and cellular pathways, particularly, cell proliferation, differentiation and survival (Faber et al., 2009). However, while regulating these cellular pathways, miRNAs can be abnormally expressed due to defects in the miRNA biosynthesis pathway and genetic alterations (e.g., mutations and chromosomal abnormalities), (Visone and Croce, 2009). MiRNAs have been classified as tumour suppressors or oncogenes (oncomirs), depending on the cancer and cellular environment in which they are expressed (Zhang et al., 2007). Tumour suppressor miRNAs (TS-miRNA) are down-regulated in cancer; they usually prevent cancer development by inhibiting oncogenes or genes that control cell proliferation and differentiation (Kent and Mendell, 2006). Oncogenic miRNAs (oncomirs), on the other hand, are up-regulated and therefore promote the development of cancer by stimulating cell proliferation (Shenouda and Alahari, 2009).

In colorectal cancer, TS-miRNAs are commonly seen in patients who do not have lymph node metastasis, well differentiated tumours, early stages (I/II) and better prognosis, implying that TS-miRNAs play a role in preventing the progression of this cancer (Wiggins et al., 2010). Oncogenic miRNAs on the other hand, are associated with presence of lymph node metastasis, poorly differentiated tumours, advanced stages (III/IV) and poor prognosis of CRC, inferring that they drive or promote the progression of this disease (Zhang et al., 2007). MiRNAs also contribute to the development and progression of CRC by targeting and regulating biological pathways (e.g., EMT) involved in carcinogenesis (Hao et al., 2014). Investigating the expression profiles of tumour suppressor and oncogenic miRNAs may therefore be helpful in predicting progression and prognosis in individuals with CRC.

1.9. Epithelial mesenchymal transition (EMT)

Epithelial-mesenchymal transition (EMT) is a biological process in which epithelial cells lose their cell-cell adhesion role and gain mesenchymal features i.e., invasion and migration properties (Yang and Weinberg, 2008). The EMT process is therefore characterized by a loss or down-regulation of epithelial proteins and increased expression of mesenchymal transcription proteins (Lee et al., 2006).

1.10. EMT-associated transcription factors (EMT-ATFs)

EMT is triggered and regulated by various EMT-ATFs, including the zinc-finger E-box-binding homeobox (zeb), basic helix-loop-helix factors (Twist1 and Twist2) and snail family (snail1 and snail2) of zinc-finger transcription factors:. These transcription factors (TFs) recognize the E-box in the promoter region of E-cadherin, resulting in the gain of the mesenchymal phenotype i.e., N-cadherin and vimentin expression.

1.10.1. Zeb

Zeb is a zinc finger E-box-binding homeobox protein found in humans. It has two forms i.e. zeb-1 and zeb-2. Zeb-1 is encoded by the *zeb-1* gene and is located in chromosome 10p11.2. Furthermore, zeb-2 is encoded by *zeb-2* gene and is located in chromosome 2q22.3 (Schmalhofer et al., 2009). The zeb protein connects with a transcription factor *Brahma Related Gene1* (*BRG1*), in its N terminal region. This complex then binds to the E2 box of the *CDH1* gene promoter region. Once the zeb/BRG1 complex binds to the *CDH1* E2 box, it changes the DNA structure, thereby suppressing the E-cadherin protein expression (Sanchez-Tillo et al., 2010).

1.10.2. Twist

Twist, also referred to as class A basic helix-loop-helix protein 38 (bHLH38) is encoded by the *twist* gene. This protein has two types, i.e., twist-1(located in chromosome 2p21.2) and twist-2 (chromosome 2q37.3). The structure of twist is made up of two domains i.e. the C and N terminal domains (Barnes and Firulli, 2009). The N terminal region has a basic domain with amino acids that bind to the E box of *CDH1* (Margetts, 2012), whilst the C terminal region has the HLH domain which attaches to other proteins to form complexes. These proteins also have 2 α-helices in their structure, one in the C terminal region and another in the N terminal region (Jones, 2004).

1.10.3. Snail

Snail belongs to a family of basic helix-loop-helix (bHLH) transcription factors which regulate neural crest development and tumour invasion (Zavadil et al, 2008). The snail family has three members i.e. snail-1 (chromosome 20q13.13), snail-2 also known as slug (chromosome 8q11) and snail-3 (chromosome 16q24.3). These proteins interact with various EMT factors such as claudins, sox9 and fosd3. Snail-1 and snail-2 are significant regulators of EMT. They act by repressing E-cadherin expression, resulting in the up-regulation of N-cadherin protein. Their structure consists of C terminal, N terminal and Snag domains (Figure 1.5). The N terminal region is important for the transcriptional repressor activity. Snail-1 has a regulatory domain that has a nuclear export signal and a destructive box domain. However, snail-2 has a slug domain which mediates repression of epithelial proteins (Villarejo et al., 2014).

Furthermore, the C terminal region of snail-1 and snail-2 proteins consists of a DNA-binding domain (DBD), which identifies and binds to the E2-box of *CDH1*. The DBD has 4 to 6 zinc fingers (ZFs) consisting of short tandem repeats with 22 to 25 amino acids. Snail-1 has 4 ZFs

(ZF1-ZF4) and snail-2 5 ZFs (ZF1-ZF5). These ZFs have different interactions and affinities to their target genes (Villarejo et al., 2014). Snail-1 and snail-2 decrease E-cadherin expression by binding to the E2 box promoter region of *CDH1*. The snail-1 protein uses ZF1 and ZF2, while snail-2 uses ZF3 and ZF4 to bind to the E2 box of E-cadherin, causing promoter repression. After having bound to the *CDH1* promoter, the snag domain of snail-1 recruits another complex of co-repressors consisting of histone deacetylase 1 and 2 (HDAC1 and HDAC2) and sin3A (Molina-Ortiz et al., 2012). Snail-1 and Snail-2 also trigger the expression of mesenchymal molecules e.g. vimentin, fibronectin and N-cadherin. (Peinado et al., 2004).

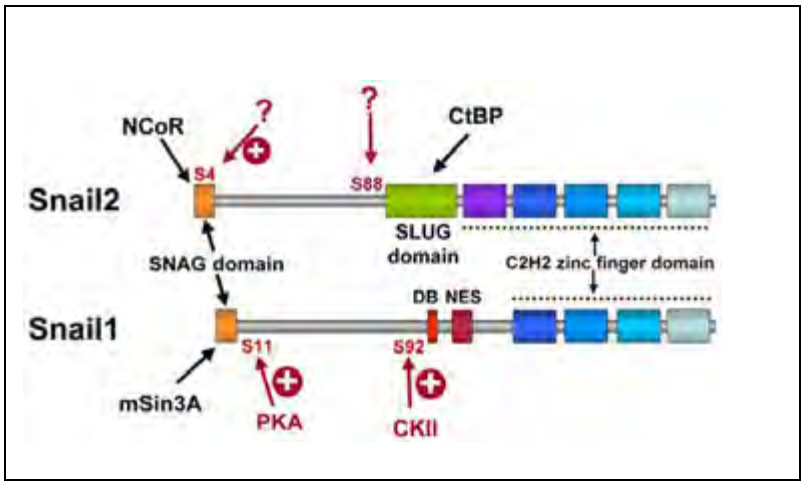


Figure 1. 5: Snail-1 and snail-2 structure consists of C terminal, N terminal and Snag domains. Snail-1 has a regulatory domain that has a nuclear export signal (NES) and a destructive box domain. Snail-2 has a slug domain which mediates repression of epithelial proteins. These proteins use the Zinc Finger (ZF) structures to bind to E-cadherin E2 box (Molina-Ortiz et al., 2012).

1.10.4. Vimentin

Vimentin is a type III intermediate filament (IF) molecule and is expressed in the cytoskeleton of mesenchymal cells. It is therefore a marker of cells going through the epithelial-mesenchymal transition process (Cabeen and Jacobs-Wagner, 2010). This protein provides stability and support to organelles in the cytosol. It also functions to sustain the cell shape and integrity of the cytoplasm. Another important function of this molecule is that it mediates cell adhesion and migration (Ivaska et al., 2007). Vimentin has three regions, namely, the non-helical amino-terminal, carboxy-terminal (tail) and α-terminal (rod) domains. The amino-terminal and carboxy-terminal make up the features of each IF molecule (Ivaska et al., 2007). The α-terminal domain has 330 amino acids and is vital for the organization of intermediate filaments (Ivaska et al., 2007; Herrmann et al., 1996).

1.10.5. N-cadherin

N-cadherin is a neural classical cadherin (N-cadherin) protein which is encoded by the *CDH2* gene. N-cadherin is a transmembrane protein with three domains, namely, the extracellular, intracellular and cytoplasmic domains (Walsh et al., 1990). The extracellular domain has five cadherin repeats (Figure 1.6), each with N and C termini. N-cadherin protein functions to mediate cell-cell adhesion in different tissues. N-Cadherin expression is commonly associated with invasion and migration of cancer cells (Araki et al., 2011).

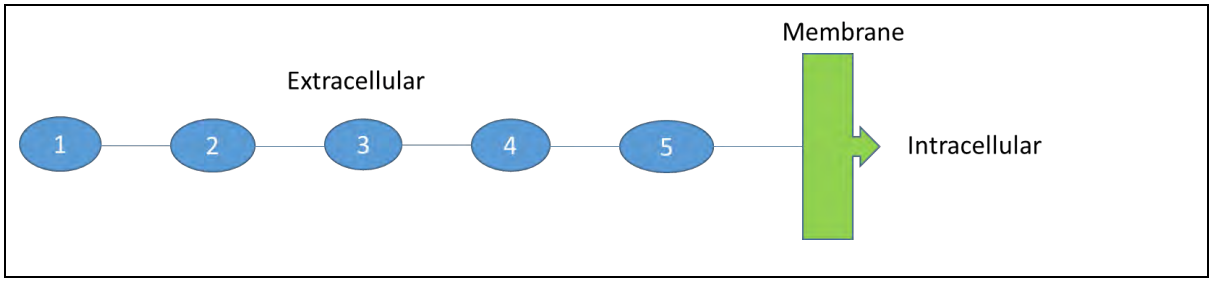


Figure 1. 6: N-cadherin protein showing extracellular, membrane and intracellular domains. The extracellular domain has five cadherin repeats, each with N and C termini.

1.10.6. E-cadherin

Epithelial (E) cadherin is a cell-cell adhesion type 1 classical transmembrane protein which is encoded by the *CDH1* gene. E-cadherin is located in the adherence junctions and has three domains, namely, the extracellular, intracellular and cytoplasmic domains (Shapiro and Weis, 2009). The extracellular domain has five cadherin repeats (Figure 1.7), each with N and C termini. In between these repeats are four calcium binding sites where calcium ions (Figure 1.7) bind (Boggon et al., 2002). These calcium ions regulate E-cadheirn function and also protect the protein from degradation (Cailliez and Lavery, 2005). The E-cadherin protein binds with various catenin molecules, namely, p120-catenin, α-catenin, γ-catenin and β-cateninin in the cytoplasmic domain to maintain cell stability (Aberle et al., 1996).

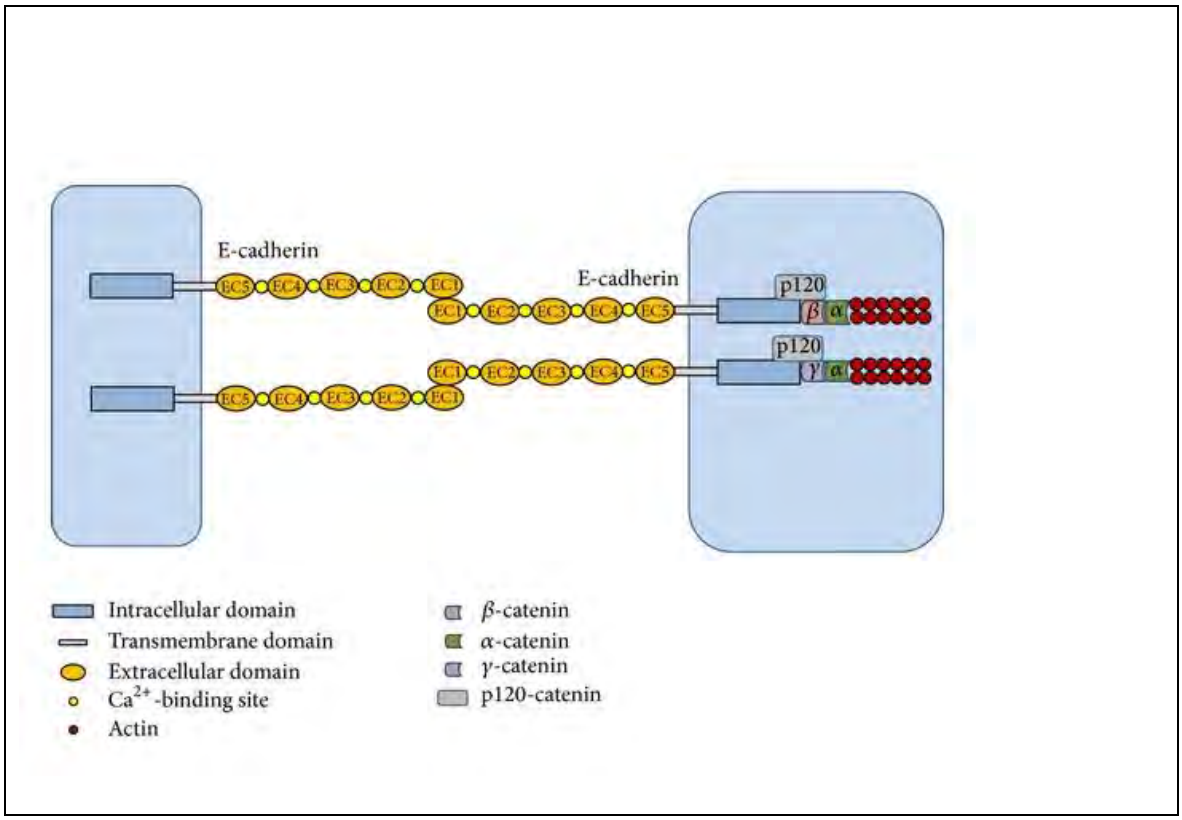


Figure 1. 7: A diagrammatic representation of the E-cadherin protein showing extracellular, transmembrane and cytoplasmic domains. E-cadherin binds with p120-catenin, α-catenin, γ-catenin and β-catenin molecules in the cytoplasmic domain (Aberle et al., 1996).

1.11. Cancer stem cell markers

Research has reported that a subgroup of cancer cells, called cancer stem cells (CSC) have the ability to stimulate cancer. CSCs were first identified in the hematopoietic model and have been reported to play a role in the development of various cancers, including colorectal cancer. Colorectal CSCs (CCSC) have been classified using CD44 or CD133 either alone or together with other markers, such as EpCAM, CD166, CD29, CD24, LGR5 and aldehyde dehydrogenase1 (ALDH1). Earlier studies have shown that CCSCs may trigger the development of CRC and that CD44 is one of the most imperative biomarkers of CCSCs.

Importantly, latest studies have demonstrated a link between EMT and CSC properties. This is supported by the expression of the EMT-markers (snail-1/snail-2 and twist) in CSC that are involved in the loss of the epithelial phenotype and the acquisition of the mesenchymal phenotype. A summary of markers which play roles in EMT and CSC phenotype is shown in Table 1.1. Stem cell markers, i.e., CD44v6, CD44v9, CD123 and CD133 have been shown to regulate the EMT process in CRC (Fan et al., 2012; Du et al., 2013; Mashita et al., 2014; Saito et al., 2013). These studies showed an inverse relationship between expression levels of the above mentioned stem cell markers and E-cadherin (Saito et al., 2013; Mashita et al., 2014). In addition, there was an association between increased expression of the stem cell markers and mesenchymal markers (N-cadherin, vimentin, twist and snail), (Fan et al., 2012; Due et al., 2013; Mashita et al., 2014). These investigations suggested that the CCSC phenotype is correlated with the EMT phenotype in CRC.

Table 1. 1: Various molecules connecting EMT and CSC phenotype

Name	**Function**	**Reference**
CD44 and CD44v6	CSC markers, play a role in migration, invasion and EMT	Du et al., 2013 Saito et al., 2013
CD133	CSC and EMT marker	Fan et al., 2012
Snail-1/2	Involved in invasion, migration and EMT process	Fan et al., 2012
Twist	EMT, invasion, migration and metastasis	Escobar-Cabrera et al., 2013
Vimentin	Biomarker of CSC and EMT features	Mashita et al., 2014
Twist	EMT, invasion, migration and metastasis	Escobar-Cabrera et al., 2013
Vimentin	CSC and EMT phenotypes	Mashita et al., 2014

1.12. miRNAs and EMT in CRC: Overview

EMT-ATFs are classified into epithelial (e.g. E-cadherin) and mesenchymal transcription factors (TFs) e.g., snail, twist and zeb. These TFs activate the EMT process by binding to the promoter region of E-cadherin, resulting in the loss or down-regulated E-cadherin expression. In addition, the TFs stimulate expression of proteins associated with the mesenchymal phenotype, namely, N-cadherin and vimentin. Loss of the epithelial (down-regulated E-cadherin) and gain of the mesenenchymal phenotype (up-regulated N-cadherin and vimentin) is associated with sinister clinicopathologic features (e.g., presence of lymph node metastasis, poor differentiation, advanced stages and poor survival), suggesting that EMT plays a role in the progression of the disease (Fan et al., 2013; Findlay et al., 2013; Kroepil et al., 2013; Toiyama et al., 2013).

In addition, miRNAs regulate the EMT process by either inhibiting or promoting the expression levels of EMT-ATFs. During carcinogenesis, miRNAs bind to the UTR regions of these TFs. After the miRNAs bind to the TFs, they either reverse or promote the EMT process, depending on the type of miRNAs (Song et al., 2013; Tanaka et al., 2013). Tumour suppressor miRNAs reverse the EMT process by suppressing the expression of mesenchymal transcription factors. This then leads to cells regaining the epithelial phenotype, which is shown by the expression of E-cadherin (Hur et al., 2013; Long et al., 2013, Paterson 2013). On the other hand, oncomirs promote and drive the EMT process by inducing the expression of mesenchymal transcription factors. In addition, oncomirs down-regulate E-cadherin expression by targeting *CDH1* (Xiong et al., 2013; Yu et al., 2013).

1.12.1. Tumour suppressor miRNAs

Tumour suppressor miRNAs are commonly known to promote the epithelial phenotype in CRC by targeting and suppressing mesenchymal transcription factors. This implies that high expression of TS-miRNAs is associated with up-regulated E-cadherin and down-regulated expression of mesenchymal factors. This indicates that TS-miRNAs are inhibitors of EMT, suggesting their role in preventing tumour development and progression. The most frequently investigated TS-miRNAs in cancer are the miRNA-200 and miRNA-34 families.

1.12.1.1. miRNA-200 family

The miRNA-200 family consists of five molecules: miRNA-200a, miRNA-200b, miRNA-200c, miRNA-141 and miRNA-429 (Ahmad et al., 2011; Uhlamann et al., 2010). Four of the miRNA-200 family members (i.e., miRNA-200a, miRNA-200b, miRNA-200c and miRNA-141) have been shown to have an inverse relationship with N-cadherin, vimentin, Twist2, Zeb1 and Zeb2 in CRC (Long et al., 2013; Paterson et al., 2013; Hur et al., 2013). These miRNA-200 members repress the expression levels of these factors, thereby reversing EMT. Reversing the EMT process is an indication that the epithelial phenotype (increased E-cadherin expression) is promoted while the mesenchymal phenotype is inhibited (Long et al., 2013; Paterson et al., 2013; Hur et al., 2013). Two (miRNA-200c and miRNA-141) of the miRNA-200 family do not act as tumour suppressors; but rather as oncogenes, since they were shown to be up-regulated in liver metastases of CRC compared to the primary tumours (Hur et al., 2013). Therefore, further research is needed to determine whether miRNA-200c and miRNA-141 act as tumour suppressors or oncogenes in this tumour.

1.12.1.2. miRNA-34 family

MiRNA-34 family consists of three members i.e. miRNA-34a, miRNA-34b and miRNA-34c. Amongst this family, miRNA-34a which is located in chromosome 1p36 is the most commonly investigated member in CRC. MiRNA-34a targets numerous transcription factors (e.g., oncogenes and cancer stem cell markers) including CD44, CDK4, CDK6, c-Met, Notch-1, Notch-2, SIRT1 and DLL1 (Saito et al., 2015). Several studies (Nugent et al., 2012; Aherne et al., 2014; Ma et al., 2014) measured miRNA-34a expression levels in CRC. In addition, they observed an association between miRNA-34a and clinicopathologic features (Nugent et al., 2012; Ma et al., 2014). The expression pattern of miRNA-34a in CRC has been controversial. While some studies observed low expression, others observed high expression of miRNA-34a in the tumour compared to normal tissue. The majority of these studies observed that miRNA-34a expression levels were associated with better prognosis, decreased cancer cell growth, migration, invasion and metastasis in CRC. However, Nugent et al (2012) did not observe any association between miRNA-34a expression levels and clinicopathologic features.

Furthermore, there have only been two investigations that determined the relationship between miRNA-34a expression levels and EMT in CRC. MiRNA-34a expression levels were measured in cell lines (HK-2, SW480 and SW620) with EMT features, i.e., increased snail, zeb1, vimentin and decreased E-cadherin expression. These studies showed an inverse relationship between miRNA-34a expression levels and mesenchymal transcription factors in CRC cells lines (Du et al., 2012; Rokavec et al., 2014).

1.12.2. Oncogenic miRNAs (Oncomirs)

Research has shown that over-expressed miRNAs can down-regulate a tumour suppressor or up-regulate genes involved in cell differentiation and proliferation, thereby promoting the development of cancer. These kinds of miRNAs are said to act as oncogenes (Zhang et al., 2007). Increased expression of oncomirs is associated with up-regulated expression of mesenchymal transcription proteins, implying that these miRNAs stimulate EMT. Additionally, up-regulated oncomirs are indicative of an aggressive disease and poor prognosis. The most frequently investigated oncomirs in cancer studies are miRNA-155 and miRNA-21.

1.12.2.1. miRNA-155

Two investigations showed high expression levels of miRNA-155 in CRC tissues compared with normal tissues. In addition, high miRNA-155 expression levels were associated with presence of lymph node metastasis, distant metastasis, poor differentiation, advanced stages and poor survival in CRC. High miRNA-155 expression levels were also linked with increased proliferation, migration and invasion characteristics in CRC cell lines i.e., SW480 and HT-29 (Zhang et al., 2013; Qu et al., 2015). These results infer that high miRNA-155 expression is of clinical significance and is associated with progressive CRC. Therefore, miRNA155 can be used to predict clinical and pathological features (e.g., lymph node metastasis and prognosis) in CRC.

1.12.2.2. miRNA-21

MiRNA-21 (miRNA-21) was one of the first miRNAs to be defined as an oncogene. It is encoded by *TMEM49* gene in chromosome 17q23-1. It is one of the most important miRNAs associated with the development and progression of different human cancers. MiRNA-21 targets various transcription factors (oncogenes and tumour suppressor genes), therefore causing cell migration, invasion and metastasis. These genes include programmed cell death 4 (PDCD4), phosphatase and tensin homolog (PTEN), Cell division cycle 25 homolog A (Cdc25a), reversion-inducing-cysteine-rich protein with kazal motifs (RECK), TIMP3, maspin, nuclear factor 1 B-type (NFIB), tropomyosin 1 (TPM1), sprouty 2 (SPRY2), T-lymphoma invasion and metastasis-inducing protein 1 (TIAM1), and Ras homolog gene family, member B (RHOB) (Meng et al., 2007; Zhu et al., 2008; Asangani et al., 2008; Lu et al., 2008; Sayed et al., 2008; Cottonham et al., 2010; Liu et al., 2011; Xiong et al., 2013; Yang et al., 2015).

Xiong et al (2013) and Yang et al (2015) determined the expression of miRNA-21 and the regulatory role of PTEN protein in CRC tissues. These studies showed that inhibition of miRNA-21 resulted in increased PTEN expression. In addition, down-regulated PTEN and up-regulated miRNA-21 expression were associated with an aggressive behaviour of the cancer.

Other investigations measured miRNA-21 expression levels and determined its relationship with the clinicopathologic features of the tumour (Nielsen et al 2011; Kjaer-Frifeldt et al., 2012; Toiyama et al., 2012; Xiong et al., 2013; Bullock et al., 2014; Yang et al., 2015) in CRC. These studies showed that high miRNA-21 expression levels were associated with invasion of tumour cells, poor differentiation, presence of lymph node metastasis and stages III/IV of the cancer. This suggests that miRNA-21 plays a role in the progression of the disease.

The studies by Nielsen et al (2011); Kjaer-Frifeldt et al (2012) and Toiyama et al., 2012 showed an association between increased miRNA-21 expression levels and decreased recurrence free cancer survival, suggesting the prognostic significance of miRNA-21. In addition, Nielson and Toiyama also observed an association between miRNA-21 expression and overall survival.

Another finding by Toiyama et al (2012) is that miRNA-21 also serves as a prognostic and predictive biomarker for response to chemotherapy in CRC. These data are consistent with two previous studies (Schetter et al., 2008 and Valeri et al., 2010). In addition, miRNA-21 expression is associated with therapeutic outcome to 5FU-based therapies. Valeri et al (2010) also showed that miRNA-21 induced resistance to 5FU in colon cancer cell lines by down regulating the expression of DNA repair protein MutS homolog 2 (MSH2). These investigations support the conclusion that miRNA-21 is up-regulated in colorectal cancer and that its high expression level is associated with advanced and aggressive clinical behaviour of the tumour.

Thus far, only two studies have examined the relationship between miRNA-21 expression levels and EMT in CRC. These investigations included CRC cell lines (Caco-H2, HCT116, Colo-205, Caco-2 and RKO cells) with EMT features (down-regulated E-cadherin protein and up-regulated N-cadherin/vimentin expression). MiRNA-21 expression levels were also measured in the cell lines which did not have EMT features. There were high miRNA-21 expression levels in the cell lines with EMT features compared to the cell lines with no EMT. These results inferred that this oncomir (miRNA-21) activates the EMT process, thus contributing to the progression of CRC.

1.13. Rationale for the study

EMT is regulated by miRNAs that either function as tumour suppressors or oncogenes (oncomirs). Tumour suppressor miRNAs reverse EMT while oncomirs activate it. Therefore, identifying how miRNAs regulate the EMT process is important in addressing the progression of CRC. This study will focus on investigating the expression levels of miRNA-21 and miRNA-34a in relation to the EMT phenotype. These two miRNAs are investigated since they are commonly de-regulated in cancer and said to function co-operatively (Hashimi et al., 2009; Sun et al., 2013; Ma et al., 2014). Since there has not been any study conducted on the role of miRNA and EMT in colorectal cancers in South Africa, we carried out an investigation to determine if there was a relationship between these parameters in our population cohort. The aim of this study was to evaluate the relationship between miRNAs (miRNA-21 and miRNA-34a) and EMT markers in colorectal cancer cases seen in the Western Cape. In addition, this investigation aimed to correlate miRNA and EMT data with clinicopathologic features in order to determine if the two miRNAs can be used as potential biomarkers to monitor progression.

The objectives of this study are to:

1. Classify EMT positive (high expression of mesenchymal transcription factors) and EMT negative (high expression of the epithelial protein) CRC cases using IHC.
2. Correlate the expression levels of mesenchymal markers (N-cadherin, vimentin and snail1) and a stem cell marker (CD44v6) with the epithelial protein (E-cadherin) in CRC cases.
3. Quantify the expression levels of two miRNAs (miRNA-21 and miRNA-34a) in EMT positive and EMT negative tumours, using qRT-PCR. Subsequently, correlate the miRNA and EMT data with the demographic and clinicopathologic data of the cases.

1.14. Hypothesis

MiRNA-21 and miRNA-34a regulate the expression of EMT-associated transcription factors (E-cadherin, N-cadherin, vimentin and snail) and thus contribute to CRC progression.

Chapter Two

2. Materials and Methods

2.1. Ethics Approval

Ethics approval for the project was obtained from the Human Research Ethics Committee, Faculty of Health Sciences (HREC REF: 250/2014), at the University of Cape Town.

2.2. Sample size determination

A statistician was consulted and the sample size was determined using the Power and Sample Size Program (PSSP). The number of cases selected was based on the worldwide proportion of the EMT markers expressed in colorectal cancer (Table 2.1); the acceptable power level was set at 80%.

Table 2. 1: Sample size and power determination using the proportion of E-cadherin, snail-1 and twist expressed in CRC tissue, worldwide.

EMT marker*	**Sample size (n)**	**EMT + Proportion (%)**	**EMT- Proportion (%)**	**E-cad – Repressed[$] (%)**	**E-cad + Expressed (%)**	**Power (%)**
Snail-1	50	75%	25%	87%	39%	82%
	45	75%	25%	87%	39%	78%
	30	75%	25%	87%	39%	24%
Twist	50	73%	27%	51%	23%	22%
	45	73%	27%	51%	23%	20%
	30	73%	27%	51%	23%	12%

*Snail-2 was not used to calculate power and sample size due to the low expression level of 37% in CRC tissues (Shioiri 2006).

$ The expression level of E-cadherin protein does not add up to 100% as the expression is based on a scoring system.

2.2.1. Sample collection

One hundred formalin fixed paraffin embedded (FFPE) colorectal cancer tissue blocks and their corresponding Haematoxylin and Eosin (H&E) slides were collected from the archives of the Division of Anatomical Pathology, UCT/NHLS. The H & E slides were reviewed by a pathologist (ML) and tissue blocks containing tumour and normal regions were selected. These cases included 8 HNPCCs i.e., those which were positive for *MLH1* germline mutations. The data related to these HNPCCs were obtained from the Division of Human Genetics, University of Cape Town.

2.3. Immunohistochemical staining of CRC cases

Immunohistochemistry was performed to determine the expression levels of EMT transcription factors: E-cadherin, N-cadherin, vimentin, snail-1 and CD44v6 (stem cell marker). The FFPE tissue blocks were sectioned at 3µm and picked up on coated glass slides (Histobond, Marienfeld). The 3µm tissue sections were then placed and left on a hot plate for 15 minutes to melt the wax. Subsequently, the tissue sections were incubated in 3 washes of xylene baths for 5 minutes each, to dissolve and remove wax. After dewaxing in xylene, they were incubated in 3 washes of absolute alcohol for 5 minutes each, to clear the xylene. Following the clearing process, the tissue sections were hydrated in tap water for 5 minutes. Antigen retrieval was then performed for 1 minute 30 seconds at full pressure using the appropriate retrieval buffer (Table 2.2). The tissue sections were incubated with 3% hydrogen peroxide (3% H_2O_2) for 10 minutes to block endogenous peroxidase activity. Subsequently, the tissue sections were washed in 10x phosphate buffered saline (PBS)-tween for 10 minutes to remove the blocking agent. After washing with PBS, the tissue sections were incubated with 5% normal goat serum (NGS) for 20 minutes to block background and nonspecific staining during antibody application. The tissue sections were then stained with the respective primary antibodies which were diluted and incubated as per Table 2.2. A positive control was added to each staining batch and a negative reagent control in which the primary antibody was replaced with 5% NGS was also included. Subsequent to the primary antibody incubation, a secondary polymer antibody was applied on the sections for 30 minutes at room temperature. Following secondary antibody incubation, the sections were washed in 10x PBS-tween for 5 minutes and 3,3'-diaminobenzidine (DAB) chromogen was applied for 10 minutes to visualize the staining. The sections were then counterstained with Mayer's haematoxylin, mounted with cover slips and analysed under a microscope.

Table 2. 2: Antibodies used to determine EMT status in 100 CRC cases.

Antibody	Clone	Species	Supplier	Dilution	Incubation time	Antigen retrieval	Positive control
E-cadherin	36B5	Monoclonal-mouse	Novacastra	1:20	1 hour	EDTA, pH8	Prostate
N-cadherin	IAR06	Monoclonal-mouse	Leica	1:100	30 minutes	Tris/EDTA	Testis
Vimentin	CloneV9	Monoclonal-mouse	Dako	1:600	30 minutes	Tris/EDTA	Sarcoma
Snail-1	Ab180714	Polyclonal-rabbit	Abcam	1:50	Overnight 4 ºC	EDTA, pH8	Testis
CD44v6 (Stem cell)	VFF-7	Monoclonal-mouse	Leica	1:50	1 hour	EDTA, Ph8	Tonsil

2.4. Immunohistochemical evaluation of EMT transcription factors

All the stains were evaluated together with a pathologist (M.L) and a consensus was reached on each case. It is therefore possible that there could be intra or inter observer variability. It was not possible for more people to review all the stains.

2.4.1. E-cadherin, N-cadherin and vimentin

E-cadherin expression was scored as either positive or negative. Negative expression was defined as less than 70% membranous staining of tumour cells. Positive expression, on the other hand, was defined as 70% or greater membranous staining of tumour cells. The E-cadherin scoring method was adopted from a previous study (Rubin et al., 2001). N-cadherin was scored based on the proportion of cancer cells staining; staining of < 20% of tumour cells indicated low expression and staining of ≥ 20% tumour cells indicated high expression (Nakajima et al., 2004). Moreover, membranous or cytoplasmic staining of vimentin in > 5% tumour cells was considered positive expression (Fan et al., 2013).

2.4.2. Snail-1

A semi-quantitative method was used to score snail-1. The percentage (0 to 100%) and predominant staining intensity (0 to 3) of tumour cell nuclei were analysed. The score was attained by multiplying the proportion and intensity values. A score of < 10/300 indicated negative expression of snail-1 and a score of ≥ 10/300 showed positive expression (Franci et al., 2009).

2.4.3. CD44v6

CD44v6 membrane staining of ≥ 10% tumour cells was considered positive expression (Saito et al., 2013).

2.5. Relationship between EMT markers and clinicopathologic features

The EMT and stem cell markers were correlated with clinicopathologic features of the tumour using Fisher's Exact and Pearson Chi Square tests in Stata 12. In addition, the relationship between E-cadherin and mesenchymal/stem cell markers (N-cadherin, vimentin, snail-1 and CD44v6) was determined using the same tests.

2.6. miRNA analysis

2.6.1. Case selection

Various studies have shown that a minimum sample size of 21 to 30 cases is sufficient for conducting miRNA analysis (Rentolf et al., 2011; Du et al., 2012; Xiong et al., 2013). Therefore, our study included 59 CRC cases (8 of which were HNPCCs) to quantity miRNA-21 and miRNA-34a expression levels. H&E slides were examined by a pathologist to demarcate normal and tumour regions. Subsequently, corresponding FFPE tissue blocks were sectioned at 10µm and fixed on Histobond glass slides. These 10µm sections were then dewaxed in xylene, washed in running tap water and allowed to dry at room temperature. After the sections had dried, they were superimposed onto the demarcated H & E slides, to mark off normal and tumour regions. Fifty nine tumour and 12 normal (uninvolved mucosa randomly selected as controls), (Aherne et al., 2014) sections were then scraped off and transferred into different 1.5ml Eppendorf tubes.

2.6.2. RNA Isolation from the 59 cases

Following scraping, RNA isolation was carried out using a High Pure FFPET RNA isolation Kit (Roche), according to the manufacturer's instructions. The protocol involved adding 100µl of RNA Tissue Lysis Buffer into the 1.5ml Eppendorf tubes containing the tissue sections. The Lysis buffer was added in order to rupture the cell membrane. Following that step, 16µl of 10% SDS and 16µl proteinase K were added to inactivate and remove any nucleases that might degenerate the RNA. After adding these reagents, the Eppendorf tubes were vortexed and briefly spun down at 600 rpm using a centrifuge. These Eppendorf tubes were then incubated in a water bath at 85°C for 30 minutes. Following the incubation period, 80µl of proteinase K was added onto the Eppendorf tubes which were then vortexed briefly. The sections were incubated again at 55°C for 30 minutes. After the second incubation period, a volume of 325µl

RNA binding buffer used to bind RNA and 325µl absolute alcohol (used to remove contaminants from the solution) were added to the tissue lysates. The lysates in the 1.5ml Eppendorf tubes were then briefly vortexed at 600 rpm and transferred into High Pure Filter tubes placed onto High Pure collection tubes. The tubes were then centrifuged for 30 minutes at 6, 000 x*g* and the flow through discarded. High Pure Filter tubes were then placed onto new High Pure Collection tubes and centrifuged at 16, 000 x*g* to dry the filter completely. The High Pure Filter tubes were again placed onto new collection tubes and 100µl of DNase solution used to digest residual DNA was added without touching the fleece of the filters. The High Pure Filter tubes were then incubated at room temperature for 15 minutes. Thereafter, a volume of 500µl of wash buffer I was added to the High Pure Filter tubes and then centrifuged at 6, 000 x*g* for 20 seconds. The flow through was discarded and 500µl wash buffer II added to the High Pure Filter tubes. Subsequently, the tubes were centrifuged at 6, 000 *xg* for 20 seconds and the flow through was discarded. The tubes were then centrifuged at 16, 000 *xg* for 2 minutes. After centrifugation, the High Pure Filter tubes were placed in new 1.5 ml autoclaved Eppendorf tubes and 30µl of RNA Elution buffer added to the centre of the filter tube without touching the fleece. The Eppendorf tubes were then incubated at room temperature and centrifuged at 6, 000 *xg* for a minute. The RNA in the Eppendorf tubes was then stored at -20°C.

2.6.3. RNA quantification and poly A reaction

The RNA samples were quantified in a Nanodrop (Thermo Scientific, Wellington, DE 19810 USA) instrument using a volume of 2µl. The samples were then diluted to 100ng and Poly A reaction was carried out in a final volume of 20µl consisting of template RNA (100ng); 5 x PAP buffer; 25mM $MnCl_2$; 100mM ATP; nuclease free water and 2U/µl of Poly (A) polymerase. A negative control (nuclease free water instead of RNA) was included in the reaction. The PCR conditions used were 37°C for 75 minutes and 65°C for 25 minutes.

2.6.4. cDNA synthesis

Subsequent to the poly A reaction, cDNA was synthesized in a final volume of 20µl using 100ng Poly A product, dH_2O, 10mM dNTPs, 5x transcription reverse transcriptase reaction buffer, 25mM $MgCl_2$, 40U/µl protector RNase inhibitor, 20U/µl transcriptor reverse transcriptase and 50mM adaptors. The PCR conditions were as follows:

- 42°C for 60 minutes
- 42°C for 75 minutes
- 85°C for 10 minutes

The cDNA products were then diluted to 1000ng and stored at -20°C.

2.6.5. miRNA-21 and miRNA-34a quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

A master mix was prepared using 2x Sybrgreen (KapaBiosystems), dH_2O and 10µM of the primers (Table 2.4) for miRNA-21, miRNA-34a and 18S (18S and normal tissues were used to normalise miRNA expression in each tumour sample) in separate Eppendorf tubes. The Eppendorf tubes were then briefly vortexed and a volume of 19.3µl from each master mix was added onto 96 well plates (Table 2.5 and Table 2.6). Subsequently, a volume of 0.7µl cDNA (1000ng) from each of the samples (e.g. 1 to 15 tumour samples) was added onto the 19.3µl master mix (Table 2.5 and Table 2.6) in triplicates. A blank (B), in which sterile water was added instead of cDNA was included to normalise the reaction. The 96 well plates were then covered with adhesive sealing films to prevent evaporation, leakage and contamination between the wells. The plates were then centrifuged at high speed and placed in a Light Cycler480® instrument (Roche) to perform the qRT-PCR (Table 2.3). The qRT-PCR was performed twice to verify the quantitative data generated by the instrument.

Table 2. 3: The qRT-PCR conditions for miRNA-21 and miRNA-34a.

Stage	**Number of cycles**	**Temperature (°C)**	**Time**	**Ramp (°C/s)**
Pre-incubation	1 cycle	95	5 m	4.4
Amplification	45 cycles	95 (Denaturation)	10s	4.4
		60 (Annealing)	15s	2.2
		72 (Extension)	5s	4.4
Melting curve	1 cycle	95	5s	4.4
		65	1s	2.2
		97	Continuous	0.11
Cooling	1 cycle	40	30s	2.2

Table 2. 4: All the primers were synthesised at Molecular Cell Biology (MCB), UCT.

Molecule	**Forward primer**	**Reverse primer**
miRNA-21	AACACCAGTCGATGGGTC	GGTCCAGTTTTTTTTTTTTTTTACA
miRNA-34a	AGTGGCAGTGTCTTAGCTGG	AGGGTCCAGTTTTTTTTTTTT
18S	TTTCGCTCTGGTCCGTCTTG	TTCGGAACTGAGGCCATGAT

Table 2. 5: A representation of miRNA-21 qRT-PCR performed twice in a 96 well plate.

		Sample 1 to 15											
Master mix		1	2	3	4	5	6	7	8	9	10	11	12
miRNA-21	A	1	1	1	2	2	2	3	3	3	4	4	4
18S	B	1	1	1	2	2	2	3	3	3	4	4	4
miRNA-21	C	5	5	5	6	6	6	7	7	7	8	8	8
18S	D	5	5	5	6	6	6	7	7	7	8	8	8
miRNA-21	E	9	9	9	10	10	10	11	11	11	12	12	12
18S	F	9	9	9	10	10	10	11	11	11	12	12	12
miRNA-21	G	13	13	13	14	14	14	15	15	15	B	B	B
18S	H	13	13	13	14	14	14	15	15	15	B	B	B

Table 2. 6: A representation of miRNA-34a qRT-PCR performed twice in a 96 well plate

		Sample 1 to 15											
Master mix		1	2	3	4	5	6	7	8	9	10	11	12
miRNA-34a	A	1	1	1	2	2	2	3	3	3	4	4	4
18S	B	1	1	1	2	2	2	3	3	3	4	4	4
miRNA-34a	C	5	5	5	6	6	6	7	7	7	8	8	8
18S	D	5	5	5	6	6	6	7	7	7	8	8	8
miRNA-34a	E	9	9	9	10	10	10	11	11	11	12	12	12
18S	F	9	9	9	10	10	10	11	11	11	12	12	12
miRNA-34a	G	13	13	13	14	14	14	15	15	15	B	B	B
18S	H	13	13	13	14	14	14	15	15	15	B	B	B

2.7. Significance of miRNA-21 and miRNA-34a in MSI

The clinical significance of miRNA-21 and miRNA-34a were assessed taking into account the microsatellite instability (MSI) status.

2.7.1. Determining microsatellite instability (MSI) using IHC

IHC staining for MLH1, MSH2 and MSH6 mismatch repair (MMR) proteins (Table 2.7) was performed on the Ventana platform (Ventana Benchmark XT) to distinguish between microsatellite stable (MSS) and microsatellite unstable (MSI) tumours. Cases were evaluated for MLH1, MSH2 and MSH6. Those with nuclear staining in normal and cancerous colon epithelium were classified as MSS. Moreover, cases with a negative protein expression were classified as MSI.

Table 2. 7: MMR antibodies used to determine MSI status in 59 CRC cases

Antibody	**Clone**	**Species**	**Supplier**	**Dilution**	**Incubation**	**Antigen retrieval**	**Positive control**
MLH1	M1	Monoclonal-mouse	Ventana	Pre-diluted	Platform	Platform	Lymph node
MSH2	G219-1129	Monoclonal-mouse	Cell Marque	Pre-diluted	Platform	Platform	Lymph node
MSH6	44	Monoclonal-mouse	Ventana	Pre-diluted	Platform	Platform	Lymph node

2.8. Statistical analysis

The relative quantification method was used to determine the expression levels of miRNA-21 and miRNA-34a in the tumour relative to the normal (Shi and Chiang, 2005). The expression levels were represented as $2^{-\Delta\Delta Ct}$ values = ($Ct_{tumour} - Ct_{normal}$ miRNA) - ($Ct_{tumour} - Ct_{normal}$ 18S) (Appendices D and E). MiRNA-21 and miRNA-34a expression levels were determined for EMT markers, clinicopathologic features (stage, grade, lymph nodes metastasis and tumour site), MSI and MSS tumours using the Mann-Whitney non-parametric test in GraphPad Prism 6. A p value of ≤ 0.05 indicated statistical significance.

In addition, the average $2^{-\Delta\Delta Ct}$ values calculated for miRNA-21 and miRNA-34a will be used as cut off values to classify MSI and MSS tumours into cases with high or low miRNA expression levels (Appendices G to J).

Chapter Three

3. Results

3.1. The clinicopathologic/demographic features of all the CRC cases

The majority (78%) of our cases were older than 50 years and only 22% were younger than 50 years. There were more females (57/100) than males (43/100). Furthermore, thirty three (33%) cases presented with stage I, 14% with stage II, 44% with stage III and only 9% presented with stage IV of the tumour. Concerning grade of the tumour, 22% were well differentiated (Grade 1), 68% moderately differentiated (Grade 2) and 10% were poorly differentiated (Grade 3). Only two of the poorly differentiated cases were of the signet ring subtype. Sixty four (64%) cases did not present with lymph node metastasis whilst 36% cases showed lymph node metastasis. Forty four (44%) had a tumour in the right colon and 56 (56%) in the left colon (Table 3.1).

Table 3. 1: Clinicopathologic/demographic data of the 100 CRC cases.

Age	≤ 50 = 22 > 50 = 78
Sex Ratio (Male: Female)	43:57
Stage	
I	33
II	14
III	44
IV	9
Grade	
1	22
2	68
3	10
Lymph node metastasis	
No	64
Yes	36
Site of tumour	
Right	44
Left	56

3.2. Immunohistochemistry

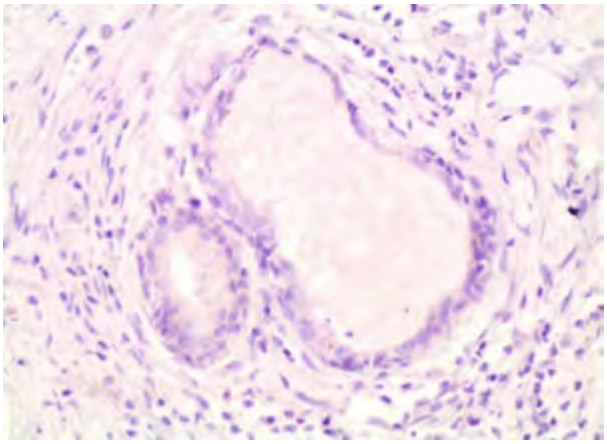
3.2.1. E-cadherin, N-cadherin, vimentin, snail-1 and CD44v6 expression levels in CRC.

There was heterogeneity of staining in different parts of the tumour. However, the percentage staining was calculated on the whole quantity of tumour present for evaluation, which therefore took the heterogeneity into account**.** In addition, we specifically looked at the interface between the tumour and adjacent normal colon for an internal control for the antibodies where it was relevant.

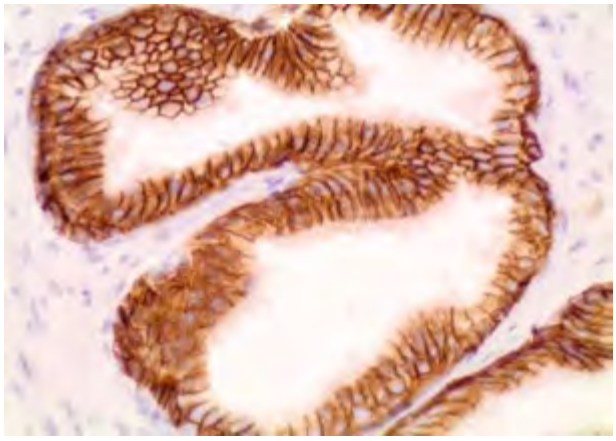
IHC stains for E-cadherin, N-cadherin, vimentin, snail-1 and CD44v6 proteins were used to ascertain EMT in our cohort. Of the 100 cases, only 23% (Table 3.2) showed membrane expression of the E-cadherin protein (Figure 3.1). In addition, 77% tumours showed down-regulated E-cadherin expression. E-cadherin was down-regulated in the cases with the signet ring morphology as well as those with no signet rings. N-cadherin and vimentin were expressed in only 2% of our cases (Table 3.2). N-cadherin was expressed in the membrane in both cases (Figure 3.2C and Figure 3.2D). Among the 2 cases that expressed vimentin, 1 showed membrane expression (Figure 3.3C) and the other one showed cytoplasmic expression. Snail-1 showed nuclear expression (Figure 3.4C) in 35% (35/100) of the cases. Lastly, membrane expression of CD44v6 (Figure 3.5C) was seen in only 22% (4/100) of the cases. These IHC data therefore show that EMT is not seen in CRCs of our cohort (Table 3.2).

Table 3. 2: Expression of EMT and stem cell markers in 100 CRC cases

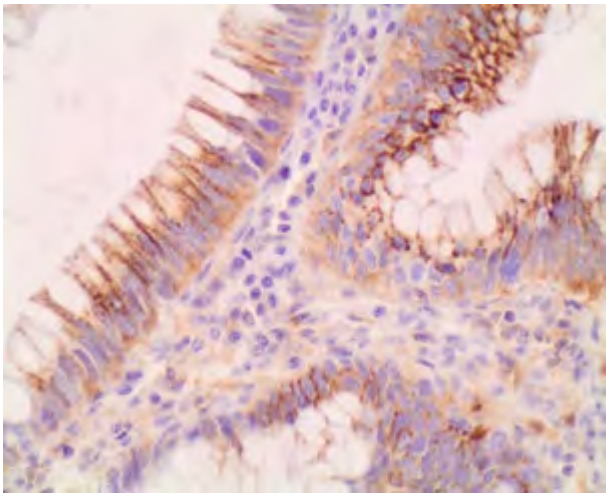
EMT marker	**Positive expression**	**Negative expression**
E-cadherin	23%	77%
N-cadherin	2%	98%
Vimentin	2%	98%
Snail-1	35%	65%
CD44v6 (stem cell)	22%	78%



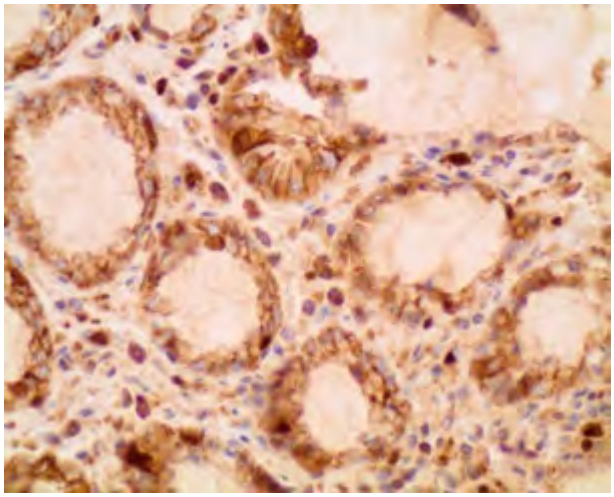
(A) E-cadherin negative control



(B) E-cadherin positive control

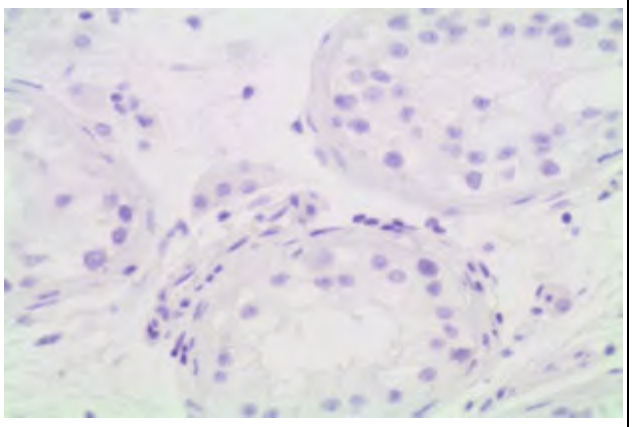


(C) E-cadherin membrane expression

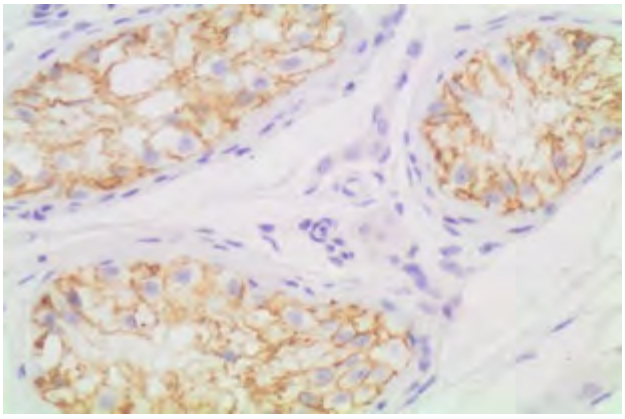


(D) E-cadherin cytoplasmic expression

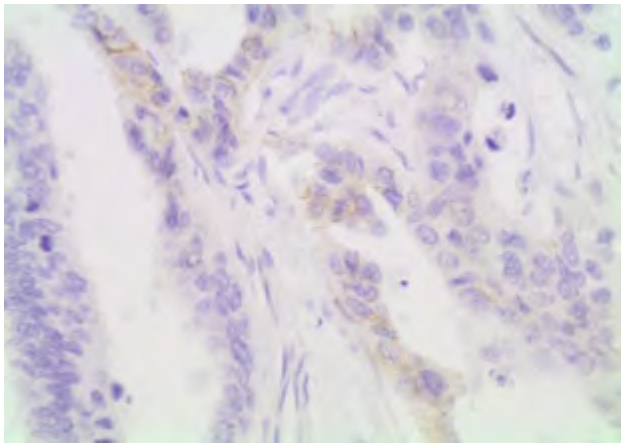
Figure 3. 1: The negative (A) and positive controls (B) are shown. IHC stains showing E-cadherin in the membrane (C) and cytoplasm (D) at 40x magnification.



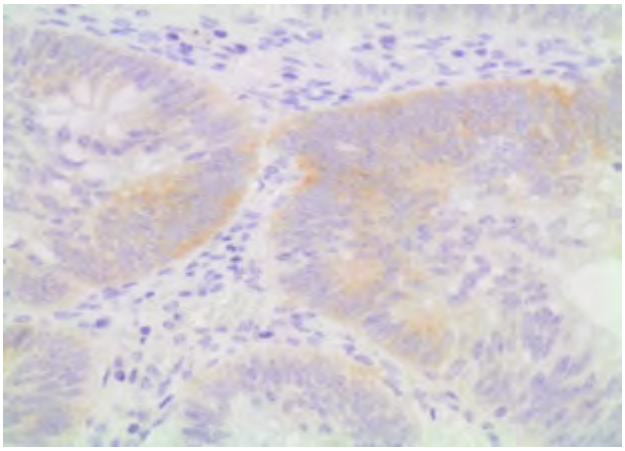
(A) N-cadherin negative control



(B) N-cadherin positive control

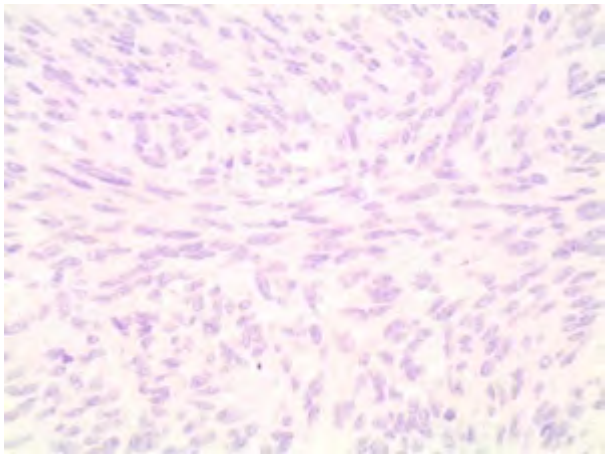


(C) N-cadherin membrane

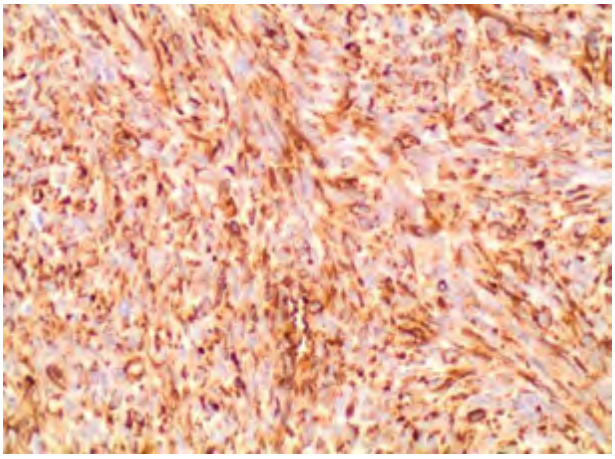


(D) N-cadherin membrane

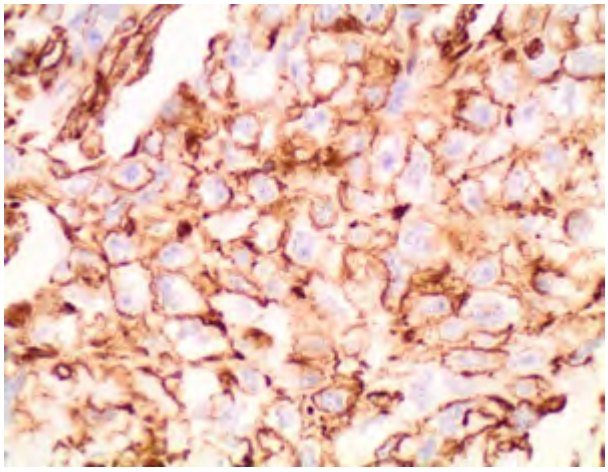
Figure 3. 2: Negative (A) and positive (B) controls are shown. IHC stains showing N-cadherin membrane expression (C) and (D) at 40x magnification.



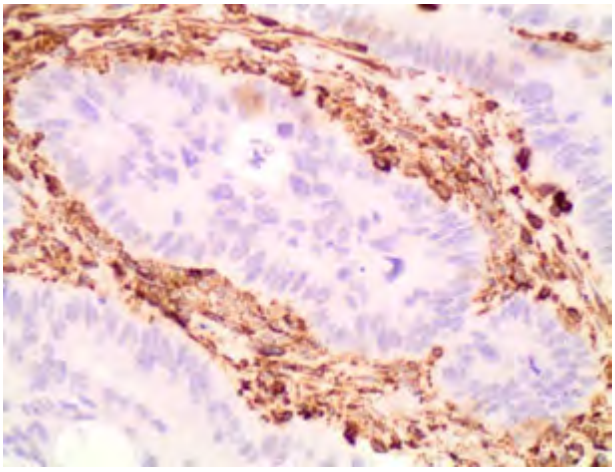
(A) Vimentin negative control



(B) Vimentin positive control

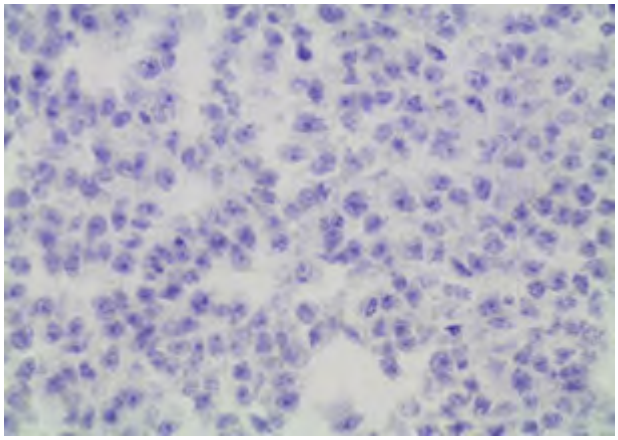


(C) Vimentin membrane expression

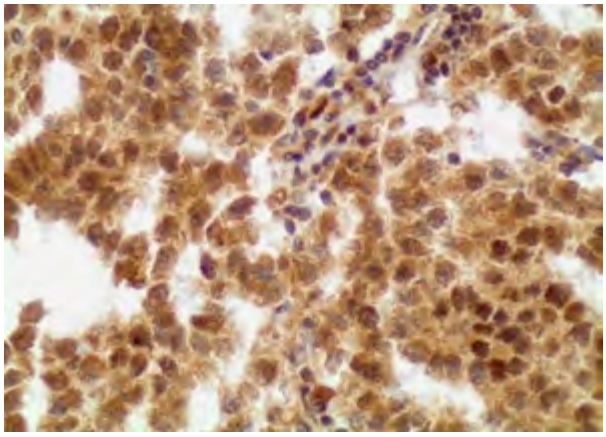


(D) Vimentin negative expression

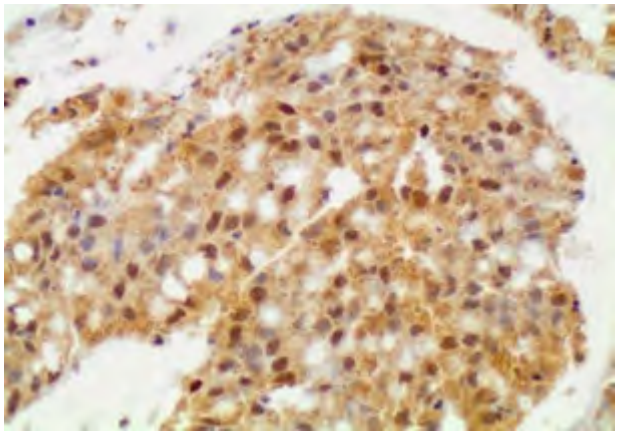
Figure 3. 3: Negative (A) and positive (B) controls are shown. IHC stains showing membrane expression (C) of vimentin. A negative expression with background staining is shown (D).



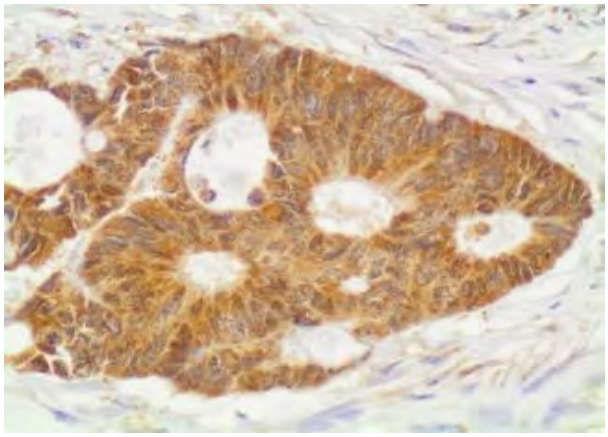
(A) Snail-1 negative control



(B) Snail-1 positive control

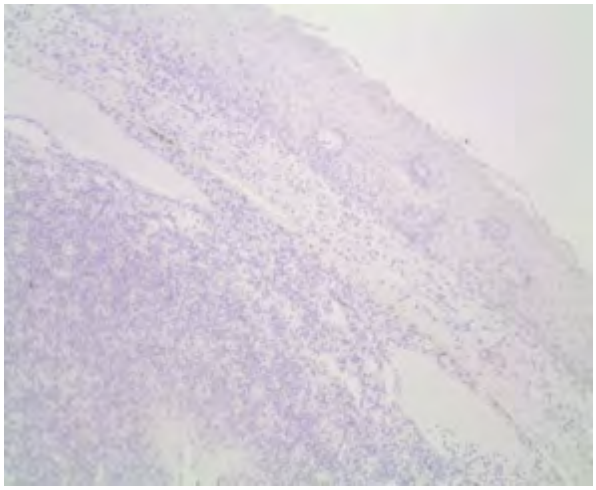


(C) Snail-1 nuclear staining

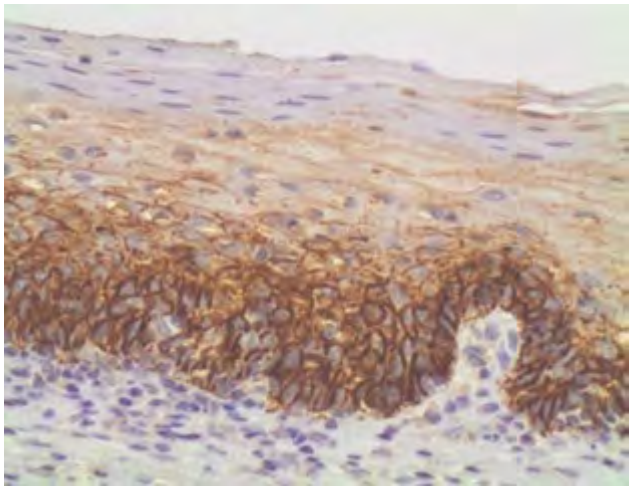


(D) Snail-1 cytoplasmic staining

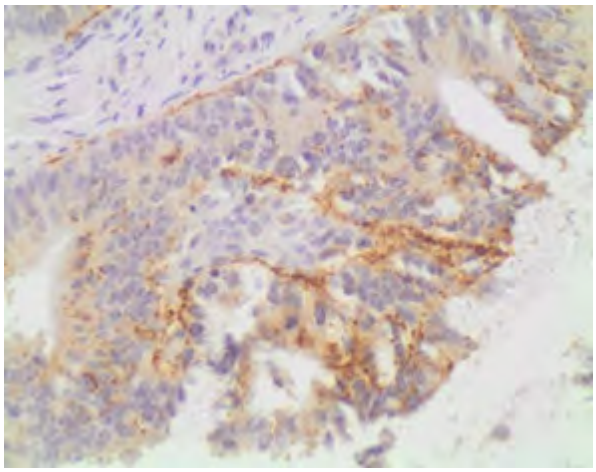
Figure 3. 4: Negative (A) and positive (B) controls are shown. IHC stains showing snail-1 nuclear (C) and cytoplasm (D) expression at 40 x magnification.



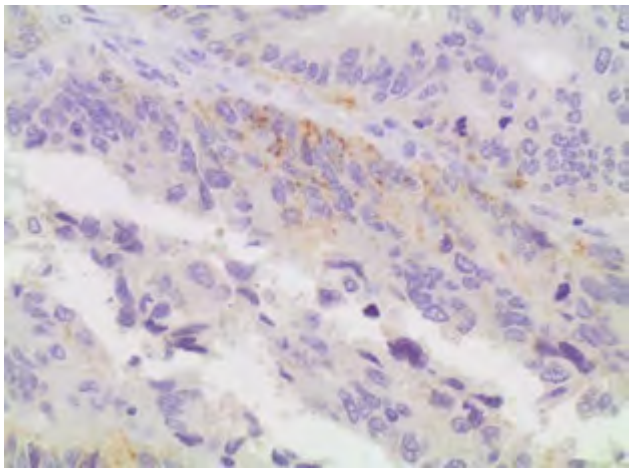
(A) CD44v6 negative control



(B) CD44v6 positive control



(C) CD44v6 membrane expression



(D) CD44v6 cytoplasmic expression

Figure 3. 5: Negative (A) and positive (B) controls are shown. IHC stains showing CD44v6 membrane (C) and cytoplasm (D) expression at 40 x magnification.

3.3. Correlations between EMT proteins and clinicopathologic features

The protein expression levels were stratified into high or low expression groups according to the scores obtained. The E-cadherin immunoreactivity categorised into negative and positive expression was not associated with any of the clinicopathologic features of the tumour (Table 3.3). Furthermore, there was no statistical significance in E-cadherin expression between stage 3 and stage 4 compared to other stages (Appendix O). There were also no significant correlations between other EMT markers (N-cadherin, vimentin, snail1 and CD44v6) and clinicopathologic data (Appendix L, I-V). Moreover, there was no relationship between E-cadherin and mesenchymal/stem cell (N-cadherin, vimentin, snail-1 and CD44v6) markers, (Appendix M, I-IV).

Table 3. 3: Relationship between E-cadherin and clinicopathologic/demographic features in 100 CRC cases.

Clinicopathologic features	E-cadherin positive expression	E-cadherin negative expression	P value
Age			0.5
≤ 50	4/22(91%)	18/22(9%)	
> 50	19/78(81%)	59/78(19%)	
Gender			0.6
Male	9/43(93%)	34/43(7%)	
Female	14/57(75%)	43/57(25%)	
Stage			0.2
I	9/33(85%)	24/33(15%)	
II	3/14(93%)	11/14(7%)	
III	7/44(77%)	37/44(23%)	
IV	4/9(89%)	5/9(11%)	
Grade			0.1
1	5/22(82%)	17/22(18%)	
2	18/68(88%)	50/68(12%)	
3	0/10(0%)	10/10(100%)	
Lymph nodes			0.8
Yes	8/36(78%)	28/36(22%)	
No	15/64(86%)	49/64(14%)	
Site of tumour			0.3
Right	8/44(80%)	36/44(20%)	
Left	15/56(86%)	41/56(14%)	

3.4. Clinicopathologic/demographic features of the 59 CRC cases used for miRNA qRT-PCR

Seventeen (29%) patients were younger or equal to the age of 50 years and 42 (71%) were older than 50 years. The cases consisted of 26 males (44%) and 33 females (56%). Fifteen cases (25%) were stage I, 7 (12%) stage II, 29 (49%) stage III and 8 (14%) stage IV. Eleven cases (19%) were well differentiated (Grade 1), 68% (40/59) moderately differentiated (Grade 2) and only 8 (14%) were poorly differentiated. Thirty four (58%) patients did not present with lymph node metastases, while 25 (42%) had at least one node affected. Thirty cases (51%) had a tumour in the right colon and 29 (49%) in the left colon (Table 3.4).

Table 3. 4: Clinicopathologic/demographic data of the 59 CRC cases used for miRNA qRT-PCR

Age	≤ 50 = 17 > 50 = 42
Sex Ratio (Male: Female)	26:33
Stage	
I	15
II	7
III	29
IV	8
Grade	
1	11
2	40
3	8
Lymph node metastasis	
No	34
Yes	25
Site of tumour	
Right	30
Left	29

Table 3. 5: Expression of EMT and stem cell markers in the 59 CRC cases used for miRNA qRT-PCR

EMT marker	**Positive expression**	**Negative expression**
E-cadherin	11/59	48/59
N-cadherin	2/59	57/59
Vimentin	1/59	58/59
Snail-1	7/59	52/59
CD44v6 (stem cell)	12/59	47/59

3.5. miRNA-21 expression levels in CRC

There is a statistically significant increase in miRNA-21 expression levels in the tumour relative to the normal tissues. The average $2^{-\Delta\Delta Ct}$ values of miRNA-21 expression for the tumour and normal tissues were 3.5 and 2.6, respectively (Appendices E and G).

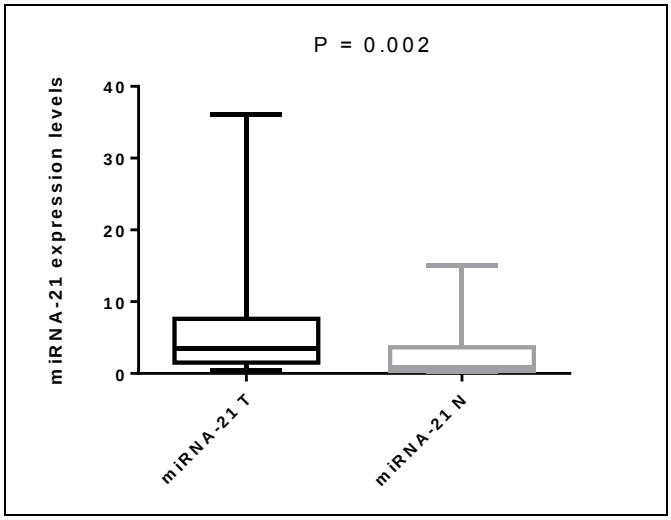
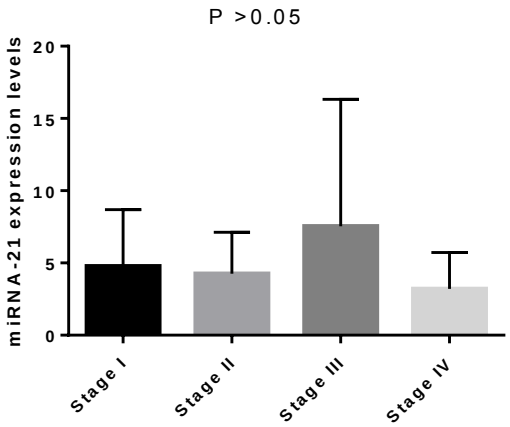


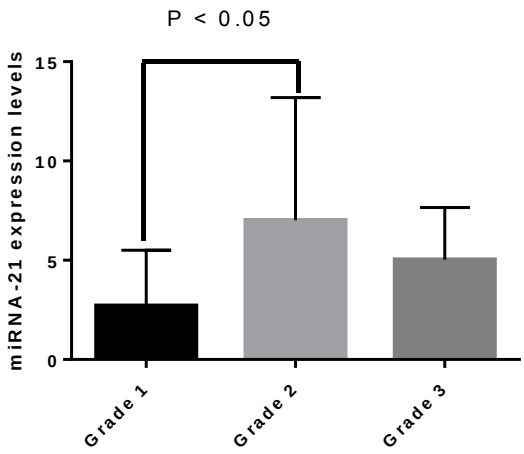
Figure 3. 6: miRNA-21 expression levels in the tumour (miRNA-21 T) relative to the normal tissues (miRNA-21 N).

3.5.1. miRNA-21 expression levels in clinicopathologic features

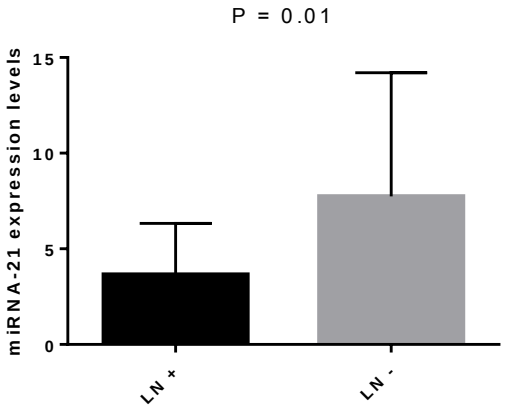
There were statistically significant differences between miRNA-21 expression levels and 2 clinicopathologic features (Figure 3.7B and Figure 3.7C) of the tumour. There was no relationship between miRNA-21 expression levels and other features (Figure 3.7A and Figure 3.7D). In addition, there were high miRNA-21 expression levels in patients older than 50 years compared to those younger or equal to 50 years of age, p=0.04 (Figure 3.8).



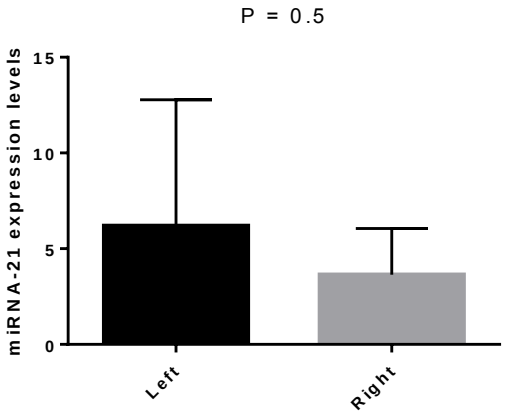
(A): miRNA-21 vs stage



(B): miRNA-21 vs grade



(C): miRNA-21 vs lymph node metastasis status



(D): miRNA-21 vs tumour site

Figure 3. 7: miRNA-21 expression levels in clinicopathologic features. (A): Stage, (B): Grade, (C): lymph node (LN) metastasis and (D): Site of tumour (Left and Right).

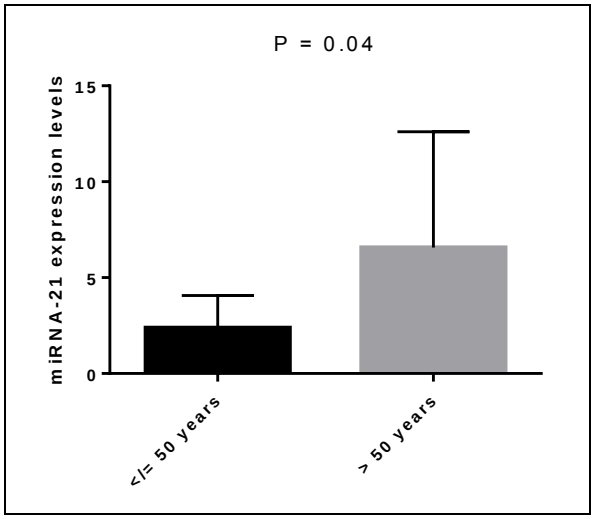
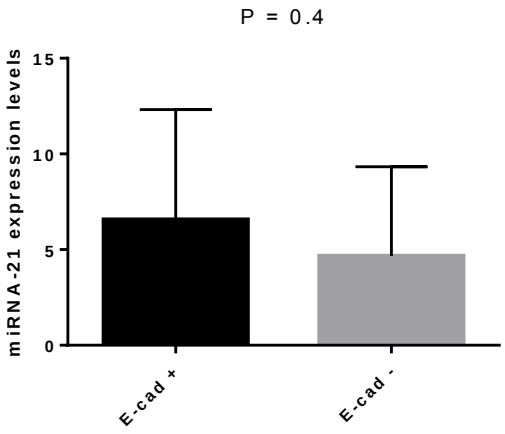


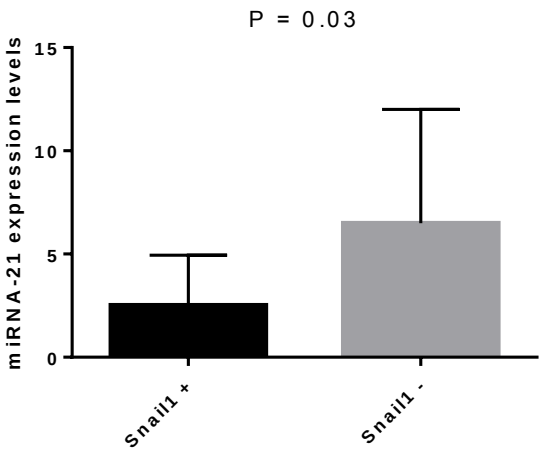
Figure 3. 8: miRNA-21 expression levels in patients equal to or younger than 50 years (</= 50 years) vs those older than 50 years (> 50 years) of age.

3.5.2. miRNA-21 expression levels and EMT in CRC

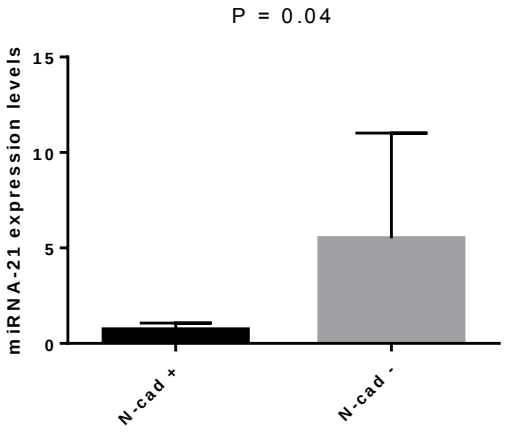
There were no significant correlations between miRNA-21 and E-cadherin expression levels (Figure 3.9A). There were significantly higher miRNA-21 expression levels in snail-1 negative cases, p=0.03 (Figure 3.9B) and N-cadherin negative cases, p=0.04 (Figure 3.9C). We could not obtain a statistical significant value for miRNA-21 and vimentin (Figure 3.9D), since there was only one individual that expressed this protein among the 59 cases (Table 3.5). There was also no significant difference between miRNA-21 and CD44v6 expression levels (Figure 3.10).



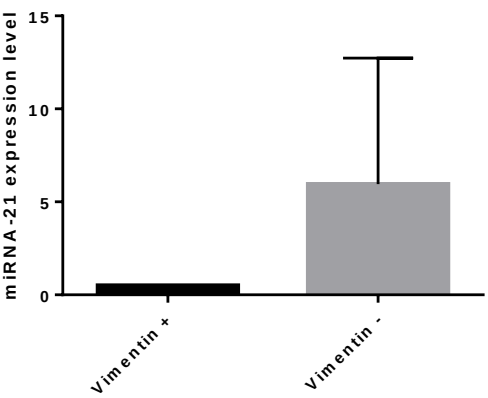
(A) miRNA-21 vs E-cadherin



(B) miRNA-21 vs Snail-1



(C) miRNA-21 vs N-cadherin



(D) miRNA-21 vs Vimentin

Figure 3. 9: (A): The differences in miRNA-21 expression levels among cases with a positive (E-cad +) and negative (E-cad -) E-cadherin protein expression; (B): miRNA-21 expression levels in snail1 positive (Snail1 +) and snail1 negative (Snail1 -) cases; (C): miRNA-21 expression levels in N-cadherin positive (N-cad +) and N-cadherin negative (N-cad -) cases and (D): miRNA-21 expression levels in vimentin positive (vimentin +) and vimentin negative (vimentin -) cases

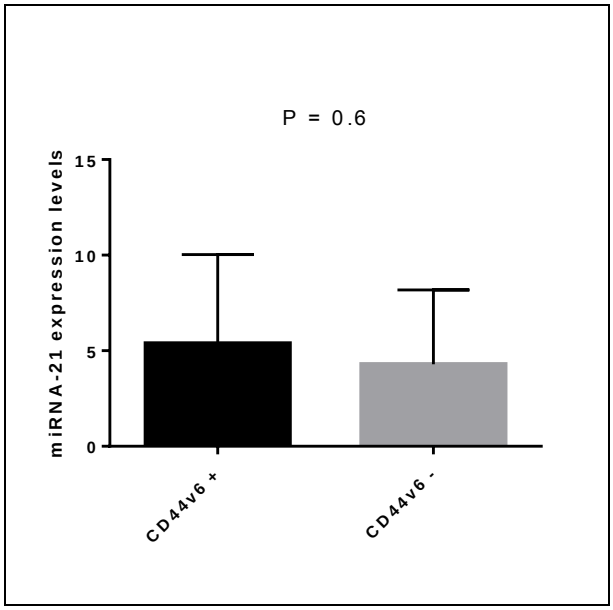


Figure 3. 10: The differences in miRNA-21 expression levels among cases with a positive CD44v6 (CD44v6 +) and negative CD44v6 (CD44v6 -) protein expression.

3.6. miRNA-34a expression levels in CRC

The results show significantly up-regulated miRNA-34a expression levels in the cancerous compared to cancerous tissues. The average $2^{-\Delta\Delta Ct}$ values for miRNA-34a expression for tumour and normal tissues were 0.6 and 0.22, respectively (Appendices E and I).

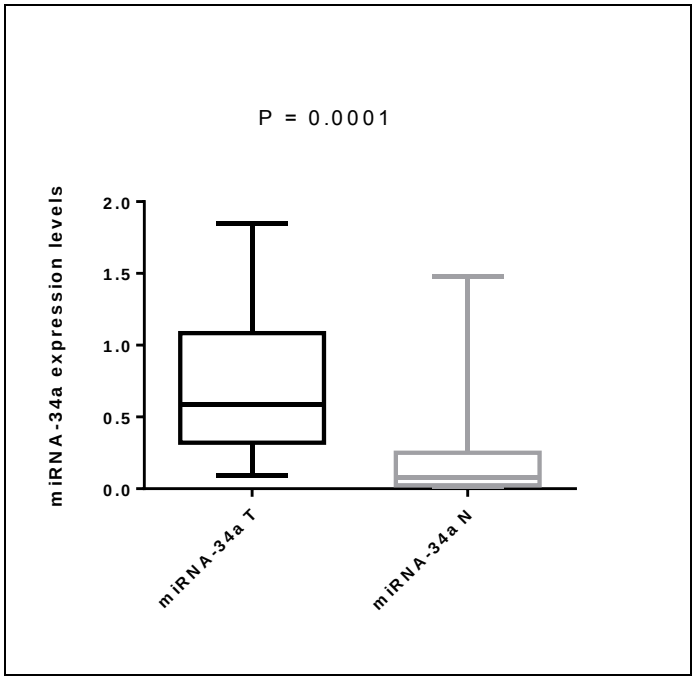
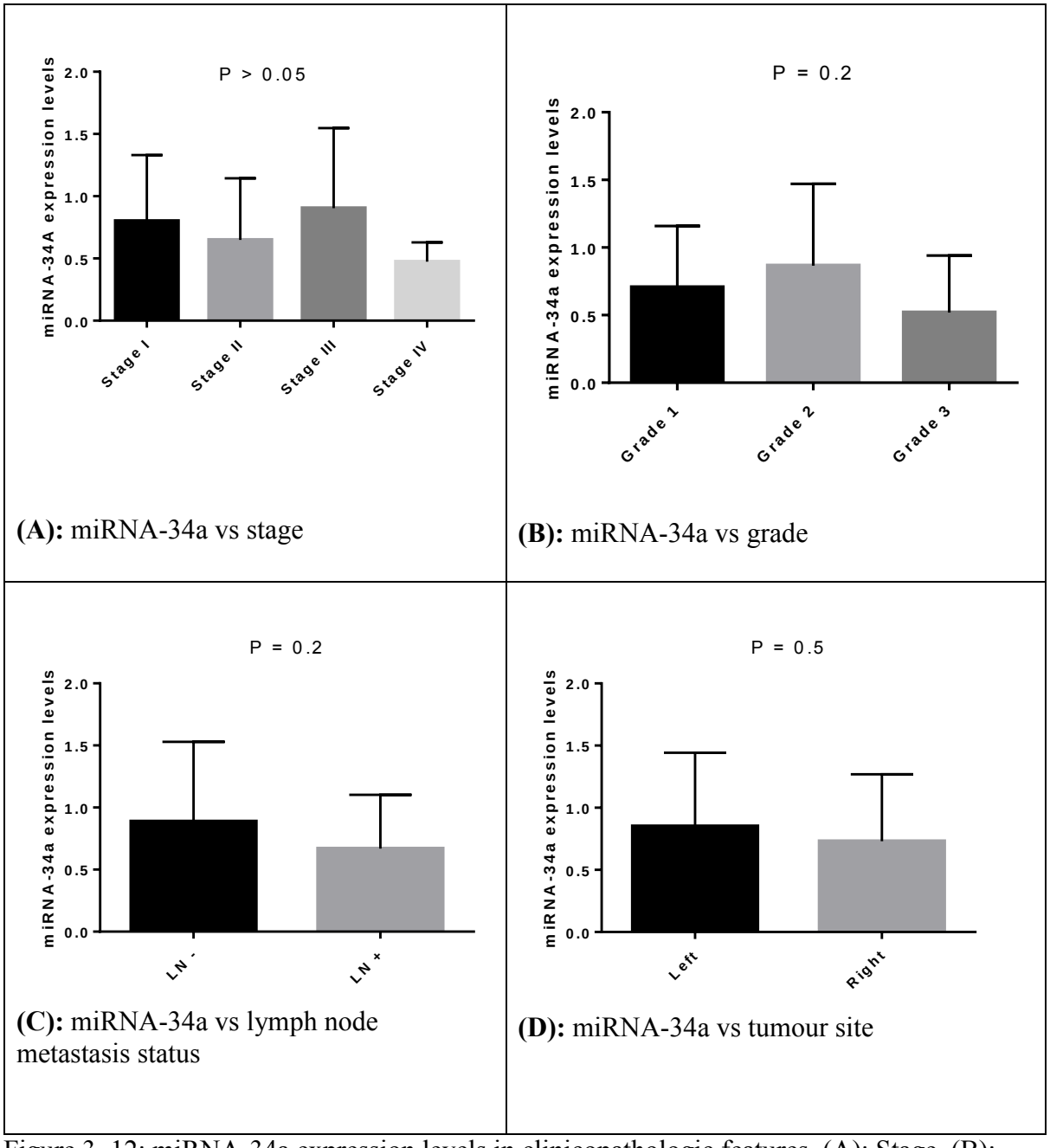


Figure 3. 11: miRNA-34a expression levels in the tumour (miRNA-34a T) relative to normal tissues (miRNA-34a N).

3.6.1. miRNA-34a expression levels in clinicopathologic features

There were no associations between miRNA-34a expression levels and clinicopathologic features of the tumour (Figure 3.12). In addition, there was no statistical significance between miRNA-34a expression levels and age (Figure 3.13).



(A): miRNA-34a vs stage

(B): miRNA-34a vs grade

(C): miRNA-34a vs lymph node metastasis status

(D): miRNA-34a vs tumour site

Figure 3. 12: miRNA-34a expression levels in clinicopathologic features. (A): Stage, (B): Grade, (C): lymph node (LN) involvement and (D): Site (Left and Right colon) of tumour.

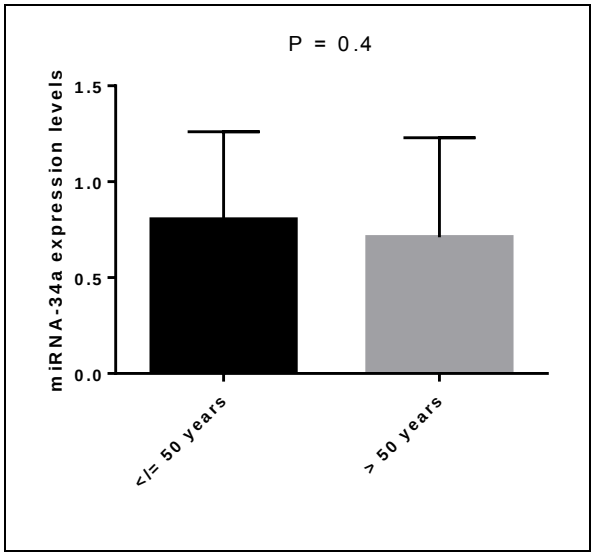
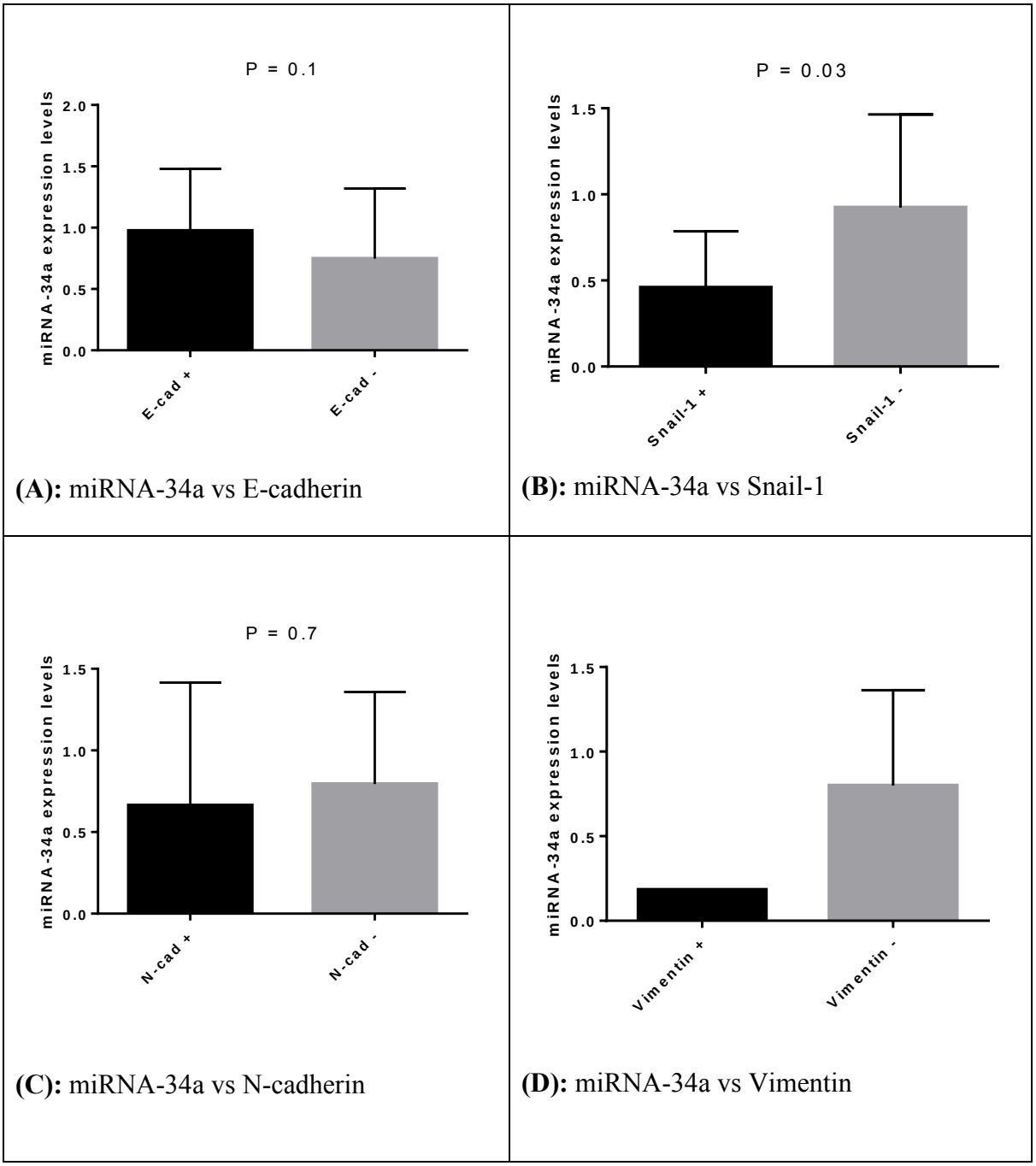


Figure 3. 13: miRNA-34a expression levels in patients younger than or equal to 50 years (</= 50 years) vs those older than 50 years (> 50 years) of age.

3.6.2. miRNA-34a expression levels and EMT in CRC

There was no statistical significance between miRNA-34a expression levels and E-cadherin protein expression, p=0.1 (Figure 3.14A). Furthermore, no correlation was observed between miRNA-34a and N-cadherin expression levels (Figure 3.14C). Moreover, we could not get a statistical significant value for miRNA-34a and vimentin (Figure 3.14D). MiRNA-34a expression was associated with snail-1 expression, p=0.03 (Figure 3.14B). There was also a correlation between miRNA-34a and CD44v6 expression levels, p=0.05 (Figure 3.15).



(A): miRNA-34a vs E-cadherin

(B): miRNA-34a vs Snail-1

(C): miRNA-34a vs N-cadherin

(D): miRNA-34a vs Vimentin

Figure 3. 14: The differences in miRNA-34a expression levels between cases with E-cadherin negative (E-cad -) and positive (E-cad +) protein expression; (B): miRNA-34a expression levels in snail-1 negative (Snail-1) and snail-1 positive (Snail-1 +) cases; miRNA-21 expression levels in N-cadherin positive (N-cad +) and N-cadherin negative (N-cad -) cases and (D): miRNA-21 expression levels in vimentin positive (Vimentin +) and vimentin negative (Vimentin -) cases.

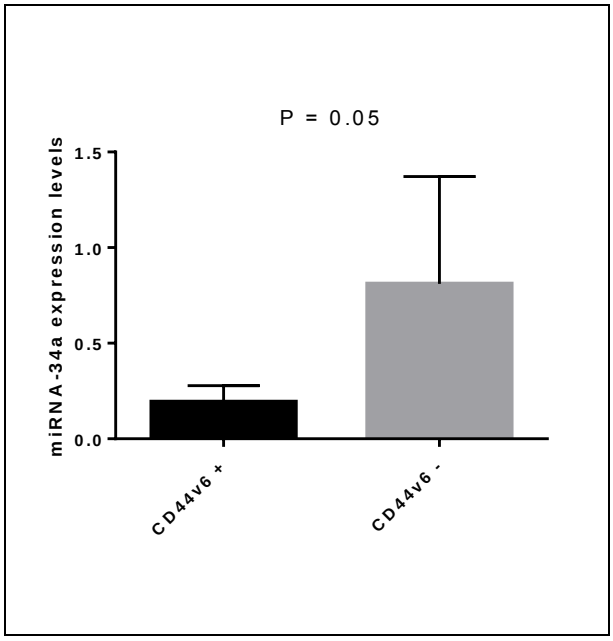


Figure 3. 15: The differences in miRNA-34a expression levels among cases with a positive CD44v6 (CD44v6 +) and negative CD44v6 (CD44v6 -) protein expression.

3.7. Significance of miRNA-21 and miRNA-34a in MSI

3.7.1. MSI and MSS tumours in 59 cases

Microsatellite unstable (MSI) tumours were defined as those which had a loss of expression of one or two mismatch repair proteins while microsatellite stable (MSS) cases expressed all the MMR proteins. Of the 51 CRC cases not previously tested for HNPCC, 5 showed loss of MLH1 expression, 5 showed loss of MSH2 expression and 6 showed loss of MSH6 expression. In addition, 7 of the 8 known HNPCC cases showed loss of MLH1 expression, 3 showed loss of MSH2 expression and 5 showed loss of MSH6 expression. Of these 59 cases, there were a total of 20 cancers with MSI (Appendix F, blue highlight).

3.7.1.1. miRNA-21 expression levels in MSI vs MSS tumours

There were higher miRNA-21 expression levels in MSS vs MSI tumours, p=0.02 (Figure 3.16). In addition, 22 of 39 MSS tumours showed high miRNA-21 expression (Appendix H). High miRNA-21 expression levels were observed in only 9 of the 20 MSI cases (Appendix G).

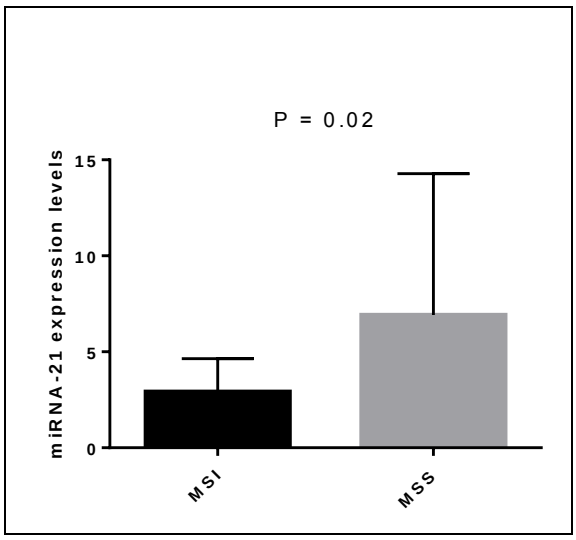


Figure 3. 16: miRNA-21 expression levels in microsatellite unstable (MSI) and microsatellite stable (MSS) tumours.

3.7.1.2. miRNA-34a expression levels in MSI vs MSS tumours

There was no significant difference in miRNA-34a expression between MSI and MSS tumours, p=0.2 (Figure 3.17). High miRNA-34a expression levels were seen in 14 of 20 MSI cases (Appendix I). Moreover, there were high miRNA-34a expression levels in 16 of the 39 MSS tumours (Appendix J).

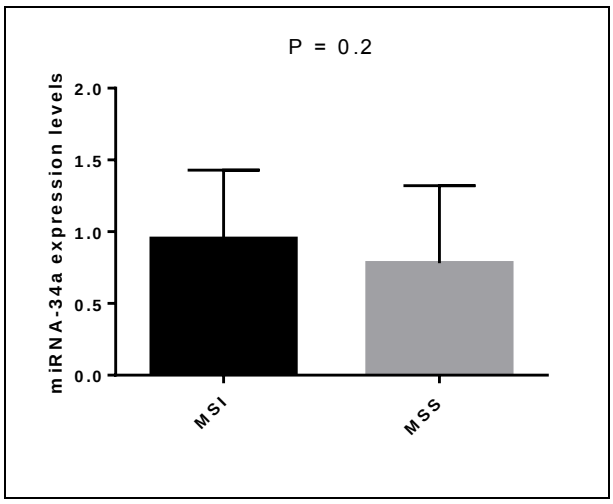


Figure 3. 17: The miRNA-34a expression levels in microsatellite unstable (MSI) and microsatellite stable (MSS) tumours.

Chapter Four

4. Discussion

4.1. Clinicopathologic and demographic features of our cohort

This study investigated the role of miRNAs and EMT in the development and progression of CRC. The cases were analysed for clinicopathologic/demographic features which included gender, age, stage, grade, tumour site and lymph node metastasis (Appendix A). Our results show higher incidence rates of CRC in females (57%) than males (43%) which is similar to published data (Globocan, 2012; Ali et al., 2014). Furthermore, CRC is frequently seen in individuals over the age of 50 (78% cases). The majority of our patients presented with stage III disease (Table 3.1) indicating advanced disease, although there were only a few individuals with stage IV tumours.

With regard to grade of the tumour, 22% were similar in appearance (well differentiated) to normal colonic glands, suggesting that the tumour cells were less aggressive. In addition, only 10% of the cases had poorly differentiated tumours. The majority (68%) of the cases had moderately differentiated tumours (Table 3.1). In addition, although one previous study reported lymph node metastasis in 67% of CRC cases (Chua et al., 2009), the present investigation shows 36% of patients having lymph node metastasis.

4.2. EMT in CRC

CRC is characterized by infiltrative growth which is associated with epithelial-mesenchymal transition (EMT). EMT is frequently seen at the invasive front of CRCs and is accompanied by an increased expression of mesenchymal markers e.g., N-cadherin and vimentin (Craene and Berx, 2013). Prerequisites for EMT are changes in the epithelial phenotype of tumour cells, i.e., the loss of epithelial polarity and gain of motility (Garg, 2013). Loss of epithelial phenotype is mediated by down-regulation of E-cadherin, a cell adhesion molecule localized at the cell membrane of epithelial cells.

In order to determine the presence or absence of the EMT phenotype, immunohistochemical analysis of 4 EMT markers was carried out and the expression levels were correlated with the clinicopathologic/demographic features. The down-regulated E-cadherin expression in 77% of cases (Table 3.2) indicates loss of the epithelial phenotype. However, only 26% (Appendix C) showed a mesenchymal feature, indicated by a positive expression of snail-1 (23 cases, green highlight); N-cadherin (1 case; yellow highlight) or vimentin (2 cases; blue highlight). It was noted that 10 of the cases which showed positive expression of E-cadherin also had up-regulated snail-1 expression (Appendix C; red highlight). This suggests that the transcription factor snail-1 did not repress E-cadherin in those cases.

It has been reported that snail-1's effectual and complete repression of *E-cadherin* as well as EMT-activating ability entails having integral ZF1 and ZF2 structures. However, alterations in the ZFs may lead to a decreased repressor activity of snail-1 (Villarejo et al., 2014). It is therefore probable that the inability of snail-1 to repress E-cadherin in the 10 cases is linked to its decreased repressor function due to molecular changes (e.g., mutations) that may have occurred. In addition, snail-1 not only represses E-cadherin but also activates the expression of

mesenchymal factors, i.e., N-cadherin and vimentin (Bezdekova et al., 2012; Zhang et al., 2013). However, all the cases that showed down-regulated E-cadherin expression and gain of snail-1 expression (green highlight) did not express N-cadherin and vimentin (orange highlight). We therefore suggest that snail-1 may have failed to activate N-cadherin and vimentin expression (mesenchymal phenotype).

Although the 23 cases possess an EMT feature (i.e., loss of E-cadherin and gain of snail-1), they cannot be deemed to have undergone a complete epithelial to mesenchymal transition, since they did not show expression of the mesenchymal phenotype (Appendix C; orange highlights). Furthermore, we suggest that 3 of the 100 cases have undergone EMT since they show both a loss of epithelial and gain of mesenchymal phenotype (Appendix C, blue and yellow highlights). However, it must be noted that these cases did not express snail-1. Therefore, our results infer that the down-regulated E-cadherin expression in those 3 cases may be explained by molecular modifications in *CDH1*. In addition, since the repression of E-cadherin also involves the participation of other EMT-ATFs (e.g., snail-2, twist and zeb) in cancer, it is possible that some of these markers may have played a role in repressing E-cadherin. Therefore, further research would have to explore and determine the expression of other EMT-TFs.

Our results did not show a relationship between E-cadherin expression levels and mesenchymal transcription factors i.e., N-cadherin, vimentin, and snail-1 (Appendix M) in this cohort. A previous study by Kroepil et al (2013) also did not observe any significant association between E-cadherin and snail-1 expression levels. However, other investigations (Fan et al., 2013; Findlay et al., 2013; Toiyama et al., 2013) showed an association between increased snail/twist and decreased E-cadherin membranous expression. This infers that these two markers induced

EMT and therefore facilitate the progression of the disease. These studies also observed high expression levels of snail/twist in more than 70% of their cases, which is different from our findings that showed 35% snail-1 expression.

Furthermore, there was no association between expression levels of all the EMT markers (E-cadherin, N-cadherin, snail and vimentin) and clinicopathologic features (lymph node metastasis, grade, stage and tumour site) in our study (Appendix L I-V). E-cadherin expression did not correlate with lymph node metastasis, grade and stage. This implies that E-cadherin is not involved in the metastatic process.

The differences between our findings and other studies regarding the expression profile of the EMT markers could be due to a number of factors. These include the different clones of antibodies used, sample type/sample size included and scoring systems. Our scoring methods (adopted from Rubin et al., 2001; Nakajima et al., 2004; Franci et al., 2009; Fan et al., 2013; Saito et al., 2013) were different from the ones used by Toiyama et al (2013) and Kroepil et al (2013). In the study by Toiyama et al (2013), vimentin expression was determined by two pathologists using an immune-reactivity scoring system through multiplying intensity and proportion scores, while E-cadherin expression was determined using qRT-PCR. Findlay et al (2013) also used qRT-PCR to determine E-cadherin, vimentin, snail and slug expression levels. Furthermore, Kroepil et al (2013) used Blechschmidt's semi-quantitative method to determine E-cadherin and snail expression (Blechschmidt et al., 2008). This is the first study to investigate the role of EMT in our population. Therefore, further research in this area will contribute to the body of knowledge.

4.2.1. EMT and CD44v6 in CRC

EMT is also related to the cancer stem cell phenotype and the presence of these cancer stem cells is associated with metastasis (Fan et al., 2012; Du et al., 2013; Mashita et al., 2014; Saito et al., 2013). Our study showed low expression of CD44v6 in 78% of the cases (Table 3.2). This is an indication that cancer stem phenotype was not seen in this cohort. Whilst there were no significant correlations between CD44v6 and EMT markers (Appendix N), our results showed an inverse relationship between CD44v6 and E-cadherin expression in 35 cases (Appendix B, purple and black/white highlights). Twenty one of these cases expressed CD44v6 while E-cadherin was down-regulated (Appendix B, black/white highlight). The remaining cases (i.e., 14 cases) showed E-cadherin expression while CD44v6 was not expressed (Appendix B, purple).

These findings concur with previous studies which also showed a similar correlation (Saito et al., 2013; Mashita et al., 2014). We also observed a concurrent expression of CD44v6 and E-cadherin in 8 cases (Appendix B, pink highlight). Furthermore, 3 of the 8 cases also showed snail-1 expression. Previous investigations reported that the stem cell and EMT phenotypes play a role in the advancement of this cancer, however, in our study these phenotypes are absent in more than 75% cases. Therefore, since EMT involves a loss of epithelial phenotype with a gain of mesenchymal and stem cell phenotypes, our results indicate that EMT is not prevalent in CRCs seen in our population.

4.3. MiRNA expression levels in colorectal cancer

MiRNA dysregulation is a common feature of human cancers as they control the expression of oncogenes and tumour suppressors, thereby functioning as onco-miRNAs or tumour suppressor miRNAs (Shenouda and Alahari, 2009). Thus far, the studies related to the role of miRNAs in CRC have been sparse. (Ping et al., 2014).

4.3.1. miRNA-21 in CRC

Various studies concluded that miRNA-21 acts an oncogene in CRC (Toiyama et al., 2012; Ferraro et al., 2013; Xiong et al., 2013; Yang et al., 2015). These conclusions were based on the fact that miRNA-21 was up-regulated in the cancer tissue when compared to their corresponding normal tissues. Our investigation showed similar results (Figure 3.6), supporting the suggestion regarding the oncogenic role of miRNA-21 in this disease. Furthermore, high miRNA-21 expression levels have been recently shown to be associated with poorly differentiated tumours, larger tumours, advanced stage (III, IV), poor survival, distant and lymph node metastatic CRCs, implying that this miRNA is involved in promoting metastasis and progression of the cancer (Toiyama et al., 2012; Xiong et al., 2013). Similarly, there was an association between high miRNA-21 expression and increasing grade (Figure 3.7B). Additionally, although there was no statistical significance, the increased miRNA-21 expression levels in stage III tumours (Figure 3.7A) may also be indicative of miRNA-21's potential role in the advancement of the disease (Slabby et al., 2007; Schetter et al., 2008).

There was also no statistical correlation when miRNA-21 expression was compared with tumour site (Figure 3.7D). There were high miRNA-21 expression levels in the cases that did not show lymph node metastasis (Figure 3.7C, p =0.01), suggesting that miRNA-21 is associated with low stage tumours. Furthermore, our study shows significantly increased miRNA-21 expression in older patients (Figure 3.8). Other studies thus far have failed to show this association.

4.3.2. miRNA-21 and EMT in CRC

Although research on the role of miRNA-21 in EMT is increasing in other cancers, there are only a few studies on colorectal cancer. Thus far, only two studies have investigated the role of miRNA-21 and EMT in CRC (Ferraro et al., 2013; Yue et al., 2015). Our results show an inverse association between miRNA-21 and snail-1 expression levels (Figure 3.9B, p=0.03). This infers that miRNA-21 did not activate snail-1 expression in this cohort. This study also shows that miRNA-21 does not stimulate N-cadherin (Figure 9C) and vimentin (Figure 3.9D) expression. It is uncommon for miRNA-21 to show increased expression in the tumours with a down-regulated snail-1 expression (i.e., absence of an EMT feature). Therefore, this shows that miRNA-21 does not play a role in the induction of EMT.

The results obtained from the present study are different to those published by Ferraro et al., (2013) and Yue et al., (2015). These two studies showed an association between high miRNA-21 expression and increased expression of snail-1 and N-cadherin. These findings imply that miRNA-21 plays a role in the activation of EMT and hence the progression of the cancer. Although miRNA-21 serves as an oncomir and has been found to activate EMT in different cancers, including CRC (Bornachea et al., 2012; Han et al., 2012; Chan and Wang, 2015; Sun et al., 2015), our findings infer that miRNA-21 does not seem to stimulate EMT nor contribute

to the progression of CRC in this cohort. The findings from the current study may be indicative that some of the EMT transcription factors (e.g., snail-1) are not regulated by miRNA-21.

Furthermore, our study did not show a statistical significance between miRNA-21 and E-cadherin expression (Figure 3.9A). We therefore suggest that miRNA-21 did not regulate E-cadherin expression in this cohort. These results are different from the two investigations (Ferraro et al., 2013; Yue et al., 2015) which reported elevated miRNA-21 expression levels in cell lines with down-regulated E-cadherin expression.

The inability of miRNA-21 to induce the EMT phenotype could be explained in various ways: e.g., there are transcription factors (e.g., AP-1, ETS1 and LIF) that bind and up-regulate miRNA-21 expression, causing it to stimulate EMT. Therefore, it is likely that these factors failed to stimulate miRNA-21 function. In addition, there are tumour suppressor genes (e.g., *PTEN*, *PDCD4*, *SPRY2, RECK*, *TIAM1*, *TIPM3* and *ITGβ4*) that target and block the function of miRNA-21 (Liu et al., 2011; Xiong et al., 2013; Yang et al., 2015). Therefore, it is also possible that these genes may have played a role in inhibiting miRNA-21 effects.

Ferraro and Yue showed that AP-1, ETS1 and LIF increased miRNA-21 expression levels. Increased PTEN protein expression in their study was associated with decreased miRNA-21 expression. Therefore, it was inferred that PTEN plays a role in blocking the effects of miRNA-21 in EMT. There may also be genetic variations within our population cohort which could possibly explain the differences in the miRNA expression levels.

In addition, CRC samples obtained from South Africa compared to the ones from Greece (Ferraro et al., 2013) and USA (Yue et al., 2015) may also have genetic differences. In the light of these results, the regulatory role of miRNA-21 in EMT is still unclear in CRC since there have only been three studies (including the current study) conducted so far. This is the first study to investigate the role of miRNA-21 and EMT in a South African landscape. Therefore, further research needs to be carried out in order to provide a clear understanding about the actual role of miRNA-21 and EMT in this disease.

Researchers who have investigated the role of miRNA-21 and EMT in other human cancers (breast, renal and prostate cancers) also obtained different results from my study (Han et al., 2012; Coppola et al., 2013; Cao et al., 2016; Yan et al., 2016). These investigations (from China and Italy) analysed the expression of 4 EMT markers (E-cadherin, N-cadherin, vimentin and slug) in cell lines induced with miRNA-21. In concordance with Ferraro and Yue, they also showed an association between the mesenchymal phenotype (i.e. increased N-cadherin, vimentin and snail-2 expression) and increased miRNA-21 expression. Furthermore, they indicated that high miRNA-21 expression was correlated with down-regulated E-cadherin expression. These findings implied that miRNA-21 activates EMT. Among these previous studies, Cao et al (2016) observed significantly increased miRNA-21 expression in cases with normal E-cadherin expression.

4.4. miRNA-34a in colorectal cancer

MiRNA-34a is said to act as a tumour suppressor based on its down-regulated expression in cancer, including CRC (Nugent et al., 2012; Ma et al., 2014; Aherne et al., 2014). However, our findings indicate that miRNA-34a is up-regulated in our patient cohort (Figure 3.11). Our results are similar to the findings by Aherne et al (2014). The data obtained by Nugent and Ma are different from our results. Therefore, the actual role of miRNA-34a in this cancer requires further investigation.

It is known that a single miRNA can either act as an oncogene or tumour suppressor depending on the type of tissue (Berindan-Neagoe et al., 2014). Therefore, it is possible that miRNA-34a may also have other functions in this cancer. We did not observe an association between miRNA-34a expression and clinicopathologic features (Figure 3.12). MiRNA-34a expression was not associated with stage, grade or lymph node metastasis (Figure 3.12A, B and C), implying that it does not play a role in the development and metastatic process of the tumour.

4.4.1. miRNA-34a and EMT in CRC

Although research on the role of miRNA-34a and EMT in the development of other cancers is increasing, research related to miRNA-34a in colorectal cancer is sparse. Thus far, only two investigations have determined the relationship between miRNA-34a and EMT in this disease (Du et al., 2012; Rokavec et al., 2014). The findings from our study showed a significant relationship between increased miRNA-34a and low snail-1 expression (Figure 3.14B, p=0.03). Similar to the two previous studies (Du et al 2012, Rokavec et al 2014), our findings imply that miRNA-34a may be repressing snail-1 expression, thereby inhibiting EMT in CRC. Likewise, miRNA-34a has been shown to inhibit EMT by decreasing the expression of EMT-TFs in other cancers (Sun et al., 2015; Li et al., 2016). In light of these findings, miRNA-34a may be functioning as a tumour suppressor which inhibits snail-1 expression.

We cannot make the same inference about miRNA-34a and N-cadherin (Figure 3.14C), since there was no statistical significance when these were compared. Furthermore, our study did not observe an association between miRNA-34a and E-cadherin expression (Figure 3.14A). Although there was no significant relationship, miRNA-34a was increased in the cases that showed E-cadherin expression. These data infer that miRNA-34a plays a role in regulating the EMT process.

Furthermore, Du et al (2012) also reported that miRNA-34a inhibited EMT by targeting and decreasing proteins of the notch pathway, i.e., notch1 and jagged1 proteins. Similarly, a study by Qiao et al., (2015) also indicated that miRNA-34a reversed EMT through suppressing the expression of TGF-β and Smad4. This resulted in increased E-cadherin expression levels while snail and N-cadherin expression were decreased (Qiao et al., 2015). Based on these

investigations, miRNA-34a also regulates EMT through targeting other biological pathways in CRC.

4.4.2. MiRNA-21 and miRNA-34a vs CD44v6 in CRC

MiRNAs have been implicated in the regulation of cancer stem cell related proteins. Therefore, a better perspective of the relationship between miRNAs and stem cells could aid in understanding the development and progression of CRC. MiRNA-34a was significantly increased ($p=0.05$) in the cases that showed low CD44v6 expression (Figure 3.15), suggesting that miRNA-34a may be regulating the cancer stem cell phenotype. In addition, since there is an association between stem cell and EMT phenotypes, this further infers that miRNA-34a prevents EMT in our study cohort. Similar findings were also reported in pancreatic, prostate, glioblastoma and breast cancers (Liu et al., 2011; Nalls et al., 2011). MiRNA-34a was also shown to repress stem cell features in colon, breast and glioblastoma cancers through targeting notch1 (Li et al., 2009; Bu et al., 2013; Kang et al., 2015). These findings then suggest that miRNA-34a may have a role in inhibiting metastasis and progression of these cancers. These data also provide further indication that CD44v6 is a target of miRNA-34a, however, we did not find any association between miRNA-21 and CD44v6 (Figure 3.10).

4.5. Significance of miRNA-21 and miRNA-34a in MSI

To date, there has been very little research conducted on the impact of miRNAs related to microsatellite instability (MSI). Furthermore, MSI tumours have different clinical and pathologic features from MSS tumours. Generally, patients with MSI have better survival and are less likely to have metastases than MSS tumours (Saridaki et al., 2014). It is therefore stated that MSI-associated miRNAs also have a prognostic significance (Dong et al., 2014). The current study observed a correlation between increased miRNA-21 expression levels and MSS tumours (Figure 3.16, $p=0.02$). In addition, we noticed high miRNA-21 expression in a greater proportion of MSS (56.4%; Appendix H) than MSI tumours (45%; Appendix G). These results thus suggest that further studies investigate miRNA-21 as an indicator of prognosis.

Furthermore, with regard to miRNA-34a, we did not see an association between its expression levels and MSI status (Figure 3.17, $p=0.2$). However, a greater percentage of MSI tumours (70%; Appendix I) had increased miRNA-34a expression than MSS tumours (41%; Appendix J).

4.6. Strengths and limitations of IHC in the study

This investigation determined the expression of four EMT markers using IHC. All of the cases which were used came from the same laboratory. This implies that these cases had been subjected to similar pre-analytical conditions such as formalin fixation, processing paraffin embedding and storage. Although some cases had been in the archives longer than others, they were interpreted by a single pathologist over a relatively short space of time. The various factors to consider when performing IHC include antibody selection (i.e., specific clone of the antibody), method of antigen retrieval, thickness of sectioning and blocking methods, i.e., blocking endogenous peroxidise activity (Sompuram et al., 2004). In the present study, two antibodies (vimentin and snail-1) had background and non-specific staining. Vimentin had a background staining of the stroma which was not considered as a positive result. Snail-1, on the other hand, stained the majority of the cases in the cytoplasm where we only considered nuclei staining as a positive result. Furthermore, the antibodies may have not reached the whole tissue sections while it was applied manually, as compared to an automatic method.

In addition, since formalin-fixed tissues require an antigen retrieval step before IHC staining, antigen retrieval buffers were used to unmask the antigen sites, allowing the antibodies to bind (Fowler et al., 2011). The retrieval buffers can sometimes damage the morphology of the section, which in turn affects staining, however, this did not prove to be problematic in this study. With regards to sectioning, a delay between sectioning and IHC may lead to false-negative results (Economou et al., 2014). This investigation sectioned all tissue blocks prior to performing IHC. This may have led to differences in staining as some of the stains failed to work. Moreover, the blocking step, using an appropriate buffer, was performed prior to incubating the sample with the primary antibody. Therefore, sufficient washing of the blocking buffer is critical to remove excess protein that may prevent detection of the target antigen

(Buchwalow et al., 2011). Some of these factors may have possibly played a role in the problems encountered including insufficient, non-specific and high background staining.

Although the above mentioned challenges were encountered, all the tissue blocks were cut with the same microtome ensuring equal thickness of sections. Whole tissue sections which served to overcome the problem of heterogeneity staining were used for each case rather than small tissue microarrays. The methods were optimized for each antibody prior to commencing with staining of the test sections. During optimization, we worked out the best incubation period and concentration for each primary antibody. Appropriate positive and negative controls were used for the antibodies where internal controls were present. Additionally, previously described scoring systems were used to evaluate the IHC staining where the intracellular location of staining and the type of cell were carefully considered. Staining was repeated if these did not stain properly and sections were coded to avoid bias when interpreting IHC results.

Only a few studies have been conducted on the role of miRNAs and EMT in colorectal cancer. In addition, this is the first investigation to determine the role of miRNAs and EMT in the Western Cape. Therefore, further research is required to better understand the role of miRNA-21, miRNA-34a and EMT in colorectal cancer.

- Our study showed that the majority of the cases were negative for EMT, implying that EMT is not involved in the development and progression of CRC in this cohort.
- Further, high miRNA-21 expression was associated with low expression of N-cadherin and snail-1. Therefore, miRNA-21 does not appear to activate EMT, suggesting that there could be other factors that may contribute to loss of its EMT inducing activity.
- There was an association between increased miRNA-21 expression levels and grade 2 tumours, absence of lymph node metastasis and older patients.
- We also observed that increased miRNA-34a expression was significantly associated with low expression of snail-1. This infers that miRNA-34a plays a role in reversing EMT.
- There was no association between miRNA-34a expression and clinicopathologic/demographic features.
- This study also indicated an association between increased miRNA-21 expression and MSS tumours, while no correlation was observed between miRNA-34a and microsatellite status.

In conclusion, our investigation indicates that there is an inverse relationship between miRNA (miRNA-21 and miRNA-34a) expression and two EMT (N-cadherin and snail-1) markers in our colorectal cancer cohort. Our data also show that both miRNA-21 and miRNA-34a do not seem to play a role in the progression of the cancer.

Future studies and recommendations.

Although our study included 4 EMT markers, the EMT process also involves other transcription factors (TFs) which have been investigated in different cancers. Among these TFs, the most commonly studied include snail2, twist-1, twist-2, zeb-1, zeb-2 and fibronectin. Therefore, the expression profile of these markers should also be analysed when investigating EMT in CRC. Further, miRNA-21 targets various EMT-related proteins, among these are PDCD4, PTEN, SPRY2, RECK, TIAM1 and TIPM3. In addition, miRNA-34a also has numerous targets involved in EMT and the most common are notch1, notch2 and jagged1. It would thus be interesting to determine the expression profile of miRNA-21 and miRNA-34a together with these EMT related proteins. Furthermore, there are other tumour suppressor and oncogenic miRNAs involved in carcinogenesis. The most frequently investigated tumour suppressor miRNAs are the miRNA-34 (miRNA-34b and miRNA-34c) and miRNA-200 families. Since most of the research that has been carried out on miRNA-34 family focuses on miRNA-34a, the expression of miRNA-34b and miRNA-34c in relation to EMT should also be explored. In addition, the oncomiRNA miRNA-155 is one of the most frequently studied miRNAs in cancer related studies. Therefore, in view of the fact that there is no published data on the role of miRNA-155 and EMT in our population, we suggest that these be investigated in CRC. Since this study included only 59 cases to determine the relationship between miRNA expression and EMT, we recommend that future studies use a larger sample size.

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Appendices

Appendix A: Clinicopathologic/demographic features of 100 CRC cases

Case #	Age (Y)	Gender	Grade	Lymph nodes	Stage	Site of colon	Part of colon
1	65	Female	1	Yes	IV	Left	Distal
2	35	Female	3	No	II	Left	Distal
3	57	Female	1	Yes	III	Left	Distal
4	32	Female	1	Yes	III	Left	Distal
5	60	Female	3	Yes	III	Right	Proximal
6	56	Male	2	No	I	Right	Proximal
7	42	Male	1	No	I	Right	Proximal
8	62	Female	1	No	III	Left	Distal
9	51	Female	2	No	I	Left	Distal
10	83	Male	1	No	III	Left	Distal
11	65	Male	1	Yes	IV	Left	Distal
12	59	Male	2	Yes	III	Right	Proximal
13	65	Male	3	Yes	III	Right	Proximal
14	70	Female	2	Yes	I	Left	Distal
15	68	Male	2	No	II	Right	Proximal
16	51	Male	2	Yes	III	Left	Distal
17	52	Female	2	Yes	III	Left	Distal
18	50	Male	2	No	I	Left	Distal
19	72	Female	3	Yes	III	Right	Proximal
20	58	Male	2	No	I	Left	Distal
21	75	Female	1	No	II	Left	Distal
22	74	Male	2	No	III	Right	Proximal
23	50	Male	2	No	III	Left	Distal
24	59	Female	2	No	I	Right	Proximal
25	65	Female	2	No	I	Left	Distal
26	61	Female	2	No	I	Right	Proximal
27	51	Female	2	No	IV	Left	Distal
28	63	Female	2	No	III	Right	Proximal
29	60	Female	2	No	I	Left	Distal
30	70	Male	2	No	III	Right	Proximal
31	64	Male	2	No	III	Left	Distal
32	65	Male	2	No	III	Left	Distal
33	80	Male	2	No	I	Left	Distal
34	77	Male	2	No	I	Right	Proximal
35	55	Male	2	Yes	III	Left	Distal
36	60	Female	2	Yes	IV	Right	Proximal
37	73	Male	2	No	II	Right	Proximal
38	84	Female	2	No	III	Left	Distal
39	40	Male	2	No	I	Right	Proximal

40	30	Female	2	No	I	Right	Proximal
41	76	Female	2	No	III	Right	Proximal
42	53	Female	3	Yes	III	Right	Proximal
43	83	Male	1	Yes	III	Right	Proximal
44	73	Female	3	Yes	III	Right	Proximal
45	84	Female	2	Yes	III	Left	Distal
46	71	Male	2	Yes	III	Right	Proximal
47	48	Male	2	No	I	Left	Distal
48	72	Female	2	No	III	Right	Proximal
49	68	Female	2	Yes	IV	Left	Distal
50	75	Female	2	Yes	IV	Right	Proximal
51	46	Female	2	No	II	Right	Proximal
52	30	Female	2	No	II	Right	Proximal
53	22	Male	2	Yes	III	Left	Distal
54	44	Male	1	Yes	IV	Right	Proximal
55	40	Female	1	Yes	IV	Right	Proximal
56	46	Female	3	No	II	Right	Proximal
57	29	Male	3	Yes	III	Left	Distal
58	30	Female	2	Yes	III	Right	Proximal
59	49	Female	2	No	III	Left	Distal
60	33	Female	2	No	I	Right	Proximal
61	56	Male	1	No	I	Right	Proximal
62	52	Female	1	No	I	Left	Distal
63	56	Male	3	Yes	III	Right	Proximal
64	64	Male	1	No	II	Left	Distal
65	70	Female	1	No	II	Left	Distal
66	59	Female	2	Yes	III	Right	Proximal
67	50	Female	1	No	I	Left	Distal
68	67	Female	2	Yes	III	Right	Proximal
69	70	Female	1	No	I	Left	Distal
70	58	Female	2	No	II	Left	Distal
71	69	Male	2	No	II	Left	Distal
72	67	Female	2	No	I	Right	Proximal
73	58	Female	2	No	I	Left	Distal
74	58	Female	2	No	III	Right	Proximal
75	70	Female	2	No	I	Left	Distal
76	50	Female	2	No	I	Left	Distal
77	80	Male	2	Yes	III	Left	Distal
78	62	Male	2	No	IV	Left	Distal
79	67	Male	2	No	I	Left	Distal
80	61	Female	2	No	I	Left	Distal
81	60	Male	2	Yes	III	Left	Distal
82	52	Female	2	No	II	Left	Distal
83	57	Female	2	No	I	Left	Distal
84	37	Female	2	No	I	Left	Distal

85	71	Male	2	Yes	III	Left	Distal
86	66	Male	2	No	I	Left	Distal
87	61	Female	2	No	I	Left	Distal
88	68	Male	2	No	III	Left	Distal
89	68	Female	1	Yes	III	Right	Proximal
90	66	Female	3	Yes	III	Right	Proximal
91	73	Male	1	Yes	III	Right	Proximal
92	80	Female	1	Yes	III	Left	Distal
93	61	Male	2	No	I	Right	Proximal
94	26	Male	2	No	III	Right	Proximal
95	57	Female	2	No	II	Left	Distal
96	67	Male	2	No	I	Left	Distal
97	72	Male	2	Yes	III	Right	Proximal
98	67	Female	1	No	III	Right	Proximal
99	51	Male	1	No	II	Left	Distal
100	59	Female	2	No	I	Left	Proximal

Appendix B: IHC results of EMT markers

Case #	Snail-1	E-cadherin	Vimentin	CD44v6	N-cadherin
1	0	1	0	1	0
2	0	0	1	0	0
3	0	0	0	0	0
4	0	0	0	0	0
5	0	0	0	0	0
6	1	0	0	0	0
7	0	0	0	0	0
8	0	0	0	0	0
9	1	0	0	0	0
10	1	0	0	0	0
11	0	0	0	0	0
12	0	0	0	0	0
13	0	0	0	0	0
14	0	0	0	0	0
15	0	0	0	0	0
16	0	0	0	0	0
17	1	0	0	0	0
18	0	0	0	1	0
19	0	0	0	0	0
20	0	1	0	0	0
21	0	1	0	0	0
22	0	0	0	0	0
23	0	0	0	0	0
24	0	0	0	0	0
25	0	0	0	0	0
26	0	0	0	0	0
27	0	1	0	1	0
28	1	0	0	0	0
29	0	0	0	0	0
30	0	0	0	0	0
31	0	0	0	0	0
32	0	0	0	0	0
33	0	1	0	0	0
34	0	0	0	0	0
35	1	1	0	0	0
36	0	1	0	1	1
37	0	0	0	0	0
38	0	0	0	1	0
39	0	0	0	0	0
40	0	0	0	0	0
41	0	0	0	0	0
42	0	0	0	1	0

43	1	0	0	1	0
44	0	0	0	1	0
45	0	0	0	0	1
46	0	0	0	0	0
47	0	1	0	0	0
48	0	0	0	1	0
49	0	0	0	0	1
50	0	1	0	1	0
51	0	0	0	0	0
52	0	1	0	0	0
53	0	0	0	1	0
54	0	0	0	0	0
55	0	0	0	0	0
56	0	0	0	0	0
57	0	0	0	0	0
58	0	1	0	1	0
59	0	0	0	0	0
60	0	0	1	1	0
61	1	0	0	0	0
62	1	1	0	0	0
63	1	0	0	1	0
64	1	1	0	0	0
65	1	0	0	0	0
66	1	0	0	0	0
67	1	0	0	0	0
68	0	0	0	1	0
69	0	0	0	0	0
70	0	0	0	1	0
71	1	0	0	1	0
72	1	1	0	0	0
73	1	0	0	0	0
74	0	0	0	0	0
75	1	0	0	0	0
76	1	0	0	0	0
77	1	0	0	1	0
78	1	0	0	1	0
79	0	0	0	1	0
80	1	1	0	0	0
81	1	1	0	1	0
82	0	0	0	1	0
83	0	1	0	0	0
84	0	0	0	1	0
85	1	1	0	0	0
86	1	0	0	0	0
87	1	1	0	0	0

88	1	0	0	0	0
89	0	0	0	1	0
90	1	0	0	0	0
91	1	0	0	1	0
92	1	0	0	0	0
93	1	0	0	1	0
94	1	1	0	1	0
95	0	0	0	0	0
96	0	0	0	1	0
97	1	1	0	0	0
98	1	1	0	1	0
99	0	0	0	1	0
100	1	1	0	0	0
Expression level	Positive expression = 1				
	Negative expression = 0				

Appendix C: CRCs with EMT features with an asterisk (*)

Case #	Snail-1	E-cadherin	Vimentin	N-cadherin
1	0	1	0	0
2*	0	0	1	0
3	0	0	0	0
4	0	0	0	0
5	0	0	0	0
6*	1	0	0	0
7	0	0	0	0
8	0	0	0	0
9*	1	0	0	0
10*	1	0	0	0
11	0	0	0	0
12	0	0	0	0
13	0	0	0	0
14	0	0	0	0
15	0	0	0	0
16	0	0	0	0
17*	1	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	1	0	0
21	0	1	0	0
22	0	0	0	0
23	0	0	0	0
24	0	0	0	0
25	0	0	0	0
26	0	0	0	0
27	0	1	0	0
28*	1	0	0	0
29	0	0	0	0
30	0	0	0	0
31	0	0	0	0
32	0	0	0	0
33	0	1	0	0
34	0	0	0	0
35	1	1	0	0
36	0	1	0	1
37	0	0	0	0
38	0	0	0	0
39	0	0	0	0
40	0	0	0	0
41	0	0	0	0
42	0	0	0	0
43*	1	0	0	0

44	0	0	0	0
45	0	0	0	0
46	0	0	0	0
47	0	1	0	0
48	0	0	0	0
49*	0	0	0	1
50	0	1	0	0
51	0	0	0	0
52	0	1	0	0
53	0	0	0	0
54	0	0	0	0
55	0	0	0	0
56	0	0	0	0
57	0	0	0	0
58	0	1	0	0
59	0	0	0	0
60*	0	0	1	0
61*	1	0	0	0
62	1	1	0	0
63*	1	0	0	0
64	1	1	0	0
65*	1	0	0	0
66*	1	0	0	0
67*	1	0	0	0
68	0	0	0	0
69	0	0	0	0
70	0	0	0	0
71*	1	0	0	0
72	1	1	0	0
73*	1	0	0	0
74	0	0	0	0
75*	1	0	0	0
76*	1	0	0	0
77*	1	0	0	0
78*	1	0	0	0
79	0	0	0	0
80	1	1	0	0
81	1	1	0	0
82	0	0	0	0
83	0	1	0	0
84	0	0	0	0
85	1	1	0	0
86*	1	0	0	0
87	1	1	0	0
88*	1	0	0	0

89	0	0	0	0
90*	1	0	0	0
91*	1	0	0	0
92*	1	0	0	0
93*	1	0	0	0
94	1	1	0	0
95	0	0	0	0
96	0	0	0	0
97	1	1	0	0
98	1	1	0	0
99*	1	0	0	0
100	0	1	0	0

Appendix D: miRNA-21 and miRNA-34a -2ddcp expression values in 59 tumour tissues

Case no	miRNA-21 -2ddcp values	miRNA-34a -2ddcp values
1	0.447213595	0.280468849
2	0.502023	0.182773446
3	0.505782	0.331430488
4	7.027001	0.838618726
5	9.0238735	1.20701056
6	0.596356	0.688688455
7	0.510841	0.473401541
8	0.503467	0.217925202
9	0.512678	1.32951693
10	0.506518	1.443056262
11	4.899042	0.588166955
12	3.99943	0.158456149
13	5.39534	1.017750413
14	4.628848	0.345367275
15	4.3301979	0.43677141
16	5.382973	0.580359269
17	5.484393	0.323945954
18	4.587338	0.193766307
19	4.133446	0.252642727
20	8.300245	0.200699564
21	8.029385	0.944751395
22	14.9832	0.665716566
23	3.501831	0.101613809
24	7.606248	0.318078445
25	20.91673	1.765707235
26	3.6180475	0.456919499
27	14.2789265	0.51354166
28	5.4454695	1.849142609
29	8.3768305	1.016415872
30	3.3756825	0.229260366
31	12.270527	1.020448018
32	12.3269725	1.152470835
33	24.7672275	1.165962335
34	19.240004	0.545858088
35	16.648958	0.637157847
36	9.6353965	0.589193781
37	5.5076065	0.432940109
38	36.1316705	0.845930629
39	1.6044055	1.147455517
40	2.7000315	1.754503694
41	3.1607275	1.364754109

42	3.075691	2.006186649
43	2.5976345	1.738816443
44	6.7991015	0.768621111
45	0.488852	0.130960242
46	2.65453	2.489502461
47	2.278889	0.602505907
48	1.5174885	1.771025739
49	0.964083	0.184000227
50	2.39092595	0.51494735
51	2.983348	0.995246438
52	3.147416	1.454929754
53	1.071846	0.132396105
54	1.4904435	0.588134276
55	3.287223	0.532664002
56	0.845712	0.094732946
57	1.763033	1.195004624
58	0.5734565	0.758293183
59	2.521214	1.010833897

Appendix E: miRNA-21 and miRNA-34a -2ddcp expression values in 12 normal tissues

Case #	miRNA-21 -2ddcp values (cut off = 2.6)	miRNA-34a -2ddcp values (cut off = 0.22)
6	3.361301004	4.680650502
8	1.739598153	4.869799077
12	0.125462237	6.062731119
17	0.219076781	8.60953839
19	3.733035337	11.36651767
23	0.355793881	11.67789694
39	0.398609075	19.69930454
44	1.027698902	22.51384945
45	0.469944152	22.73497208
56	0.517211096	28.25860555
57	4.15103009	30.57551505
59	15.04413379	37.0220669

Appendix F: IHC results of mismatch repair proteins

Case #	MLH1	MSH2	MSH6
1	Present	Present	Present
2	Present	Present	Present
3	Present	Present	Present
4	Present	Present	Present
5	Present	Present	Absent
6	Present	Present	Present
7	Present	Absent	Absent
8	Present	Present	Present
9	Present	Present	Present
10	Present	Present	Present
11	Present	Present	Present
12	Present	Present	Present
13	Present	Present	Present
14	Present	Present	Present
15	Present	Present	Present
16	Present	Present	Present
17	Present	Present	Present
18	Present	Absent	Present
19	Present	Present	Present
20	Present	Present	Present
21	Present	Present	Present
22	Present	Present	Absent
23	Present	Absent	Present
24	Present	Present	Present
25	Present	Present	Present
26	Absent	Absent	Absent
27	Present	Present	Present
28	Absent	Present	Present
29	Present	Present	Present
30	Present	Present	Present
31	Present	Present	Present
32	Present	Present	Absent
33	Present	Absent	Absent
34	Present	Present	Present
35	Present	Present	Present
36	Present	Present	Present
37	Present	Present	Present
38	Present	Present	Present
39	Present	Present	Present
40	Present	Present	Present
41	Present	Present	Present
42	Absent	Cannot interpret	Cannot interpret

43	Absent	Present	Present
44	Absent	Present	Present
45	Present	Present	Present
46	Present	Present	Present
47	Present	Present	Present
48	Present	Present	Present
49	Present	Present	Present
50	Present	Present	Present
51	Absent	Present	Absent
52	Absent	Present	Present
53	Present	Present	Present
54	Absent	Absent	Absent
55	Present	Absent	Absent
56	Absent	Present	Present
57	Absent	Absent	Absent
58	Absent	Present	Present
59	Absent	Present	Absent

Total: 20 MSI tumours

Appendix G: miRNA-21 expression in 20 MSI tumours

Average of -2ddcp = 3.5 (cut off value)

Case #	miRNA-21 expression	miRNA-21 -2ddcp values
5	H	9.0238735
7	L	0.510841
18	H	4.587338
22	H	14.9832
23	H	3.501831
26	H	3.6180475
32	H	12.3269725
33	H	24.7672275
51	L	2.983348
52	L	3.147416
54	L	1.4904435
55	L	3.287223
56	L	0.845712
57	L	1.763033
58	L	0.5734565
59	L	2.521214
28	H	5.4454695
42	L	3.075691
43	L	2.5976345
44	H	6.7991015
H = High expression	9/20 (45%)	
L = Low expression	11/20 (55%)	

Appendix H: miRNA-21 expression in 39 MSS tumours

Average of -2ddcp = 3.5 (cut off value)

Case #	miRNA- 21 expression	miRNA-21 -2ddcp values
1	L	0.447213595
2	L	0.502023
3	L	0.505782
4	H	7.027001
6	L	0.596356
8	L	0.503467
9	L	0.512678
10	L	0.506518
11	H	4.899042
12	H	3.99943
13	H	5.39534
14	H	4.628848
15	H	4.3301979
16	H	5.382973
17	H	5.484393
19	H	4.133446
20	H	8.300245
21	H	8.029385
24	H	7.606248
25	H	20.91673
27	H	14.2789265
29	H	8.3768305
30	H	3.3756825
31	H	12.270527
34	H	19.240004
35	H	16.648958
36	H	9.6353965
37	H	5.5076065
38	H	36.1316705
39	L	1.6044055
40	L	2.7000315
41	L	3.1607275
45	L	0.488852
46	L	2.65453
47	L	2.278889
48	L	1.5174885
49	L	0.964083
50	L	2.39092595
53	L	1.071846

H = High expression	22/39 (56.4%)	
L = Low expression	17/39 (43.6%)	

Appendix I: miRNA-34a expression in 20 MSI tumours

Average of -2ddcp = 0.6 (cut off value)

Case #	miRNA-34a expression	miRNA-34a - 2ddcp
5	H	0.688688455
7	H	1.32951693
18	H	0.944751395
22	H	1.849142609
23	H	1.016415872
26	L	0.545858088
28	L	0.589193781
32	H	1.754503694
33	H	1.364754109
42	L	0.51494735
43	H	0.995246438
44	H	1.454929754
51	H	1.010833897
52	H	1.20701056
54	L	0.193766307
55	H	0.665716566
56	L	0.101613809
57	L	0.456919499
58	H	1.152470835
59	H	1.165962335
H = High expression	14/20 (70%)	
L = Low expression	6/20 (30%)	

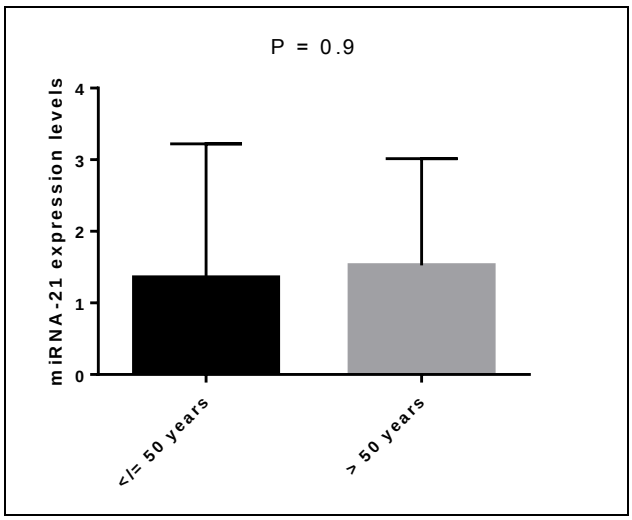
Appendix J: miRNA-34a expression in 39 MSS tumours

Average of -2ddcp = 0.6 (cut off value)

Case #	miRNA-34a expression	miRNA-34a - 2ddcp
1	L	0.280468849
2	L	0.182773446
3	L	0.331430488
4	H	0.838618726
6	L	0.217925202
8	H	1.443056262
9	L	0.588166955
10	L	0.158456149
11	H	1.017750413
12	L	0.345367275
13	L	0.43677141
14	L	0.580359269
15	L	0.323945954
16	L	0.252642727
17	L	0.200699564
19	L	0.318078445
20	H	1.765707235
21	L	0.51354166
24	L	0.229260366
25	H	1.020448018
27	H	0.637157847
29	L	0.432940109
30	H	0.845930629
31	H	1.147455517
34	H	2.006186649
35	H	1.738816443
36	H	0.768621111
37	L	0.130960242
38	H	2.489502461
39	H	0.602505907
40	H	1.771025739
41	L	0.184000227
45	L	0.132396105
46	L	0.588134276
47	L	0.532664002
48	L	0.094732946
49	H	1.195004624
50	H	0.758293183
53	L	0.132396105

H = High expression	16/39 (41%)	
L = Low expression	23/39 (59%)	

Appendix K: miRNA-21 expression vs age in the 12 normal tissues



Appendix L: EMT/stem cell markers vs clinicopathologic features (Stata 12 data)

(I) E-cadherin:

ecadherin	stage I	II	III	IV	Total
0	24	11	37	5	77
	25.4	10.8	33.9	6.9	77.0
	72.73	78.57	84.09	55.56	77.00
1	9	3	7	4	23
	7.6	3.2	10.1	2.1	23.0
	27.27	21.43	15.91	44.44	23.00
Total	33	14	44	9	100
	33.0	14.0	44.0	9.0	100.0
	100.00	100.00	100.00	100.00	100.00

Pearson chi2(3) = 3.9459 Pr = 0.267
Fisher's exact = 0.263

ecadherin	grade 1	2	3	Total
0	17	50	10	77
	16.9	52.4	7.7	77.0
	77.27	73.53	100.00	77.00
1	5	18	0	23
	5.1	15.6	2.3	23.0
	22.73	26.47	0.00	23.00
Total	22	68	10	100
	22.0	68.0	10.0	100.0
	100.00	100.00	100.00	100.00

Pearson chi2(2) = 3.4504 Pr = 0.178
Fisher's exact = 0.189

ecadherin	lymphnodemetastasis No	Yes	Total
0	49	28	77
	49.3	27.7	77.0
	76.56	77.78	77.00
1	15	8	23
	14.7	8.3	23.0
	23.44	22.22	23.00
Total	64	36	100
	64.0	36.0	100.0
	100.00	100.00	100.00

```
          Pearson chi2(1) =   0.0192   Pr = 0.890
           Fisher's exact =                 1.000
  1-sided Fisher's exact =                 0.548
```

ecadherin	siteofcolon Left	Right	Total
0	41	36	77
	43.1	33.9	77.0
	73.21	81.82	77.00
1	15	8	23
	12.9	10.1	23.0
	26.79	18.18	23.00
Total	56	44	100
	56.0	44.0	100.0
	100.00	100.00	100.00

```
          Pearson chi2(1) =   1.0299   Pr = 0.310
           Fisher's exact =                 0.347
  1-sided Fisher's exact =                 0.220

.
```

ecadherin	gender Female	Male	Total
0	43	34	77
	43.9	33.1	77.0
	75.44	79.07	77.00
1	14	9	23
	13.1	9.9	23.0
	24.56	20.93	23.00
Total	57	43	100
	57.0	43.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 0.1825 Pr = 0.669
Fisher's exact = 0.811
1-sided Fisher's exact = 0.428

(II) Snail-1:

snail1	stage I	II	III	IV	Total
0	19	11	26	8	64
	21.1	9.0	28.2	5.8	64.0
	57.58	78.57	59.09	88.89	64.00
1	14	3	17	1	35
	11.6	4.9	15.4	3.1	35.0
	42.42	21.43	38.64	11.11	35.00
0	0	0	1	0	1
	0.3	0.1	0.4	0.1	1.0
	0.00	0.00	2.27	0.00	1.00
Total	33	14	44	9	100
	33.0	14.0	44.0	9.0	100.0
	100.00	100.00	100.00	100.00	100.00

Pearson chi2(6) = 5.8769 Pr = 0.437
Fisher's exact = 0.344

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stage 1: enumerations = 0

snail1	grade 1	2	3	Total
0	12	44	8	64
	14.1	43.5	6.4	64.0
	54.55	64.71	80.00	64.00
1	10	23	2	35
	7.7	23.8	3.5	35.0
	45.45	33.82	20.00	35.00
0	0	1	0	1
	0.2	0.7	0.1	1.0
	0.00	1.47	0.00	1.00
Total	22	68	10	100
	22.0	68.0	10.0	100.0
	100.00	100.00	100.00	100.00

Pearson chi2(4) = 2.5399 Pr = 0.638
Fisher's exact = 0.583

snail1	lymphnodemetastasis No	Yes	Total
0	41	23	64
	41.0	23.0	64.0
	64.06	63.89	64.00
1	23	12	35
	22.4	12.6	35.0
	35.94	33.33	35.00
0	0	1	1
	0.6	0.4	1.0
	0.00	2.78	1.00
Total	64	36	100
	64.0	36.0	100.0
	100.00	100.00	100.00

Pearson chi2(2) = 1.8225 Pr = 0.402
Fisher's exact = 0.538

snail1	siteofcolon Left	Right	Total
0	34	30	64
	35.8	28.2	64.0
	60.71	68.18	64.00
1	22	13	35
	19.6	15.4	35.0
	39.29	29.55	35.00
0	0	1	1
	0.6	0.4	1.0
	0.00	2.27	1.00
Total	56	44	100
	56.0	44.0	100.0
	100.00	100.00	100.00

Pearson chi2(2) = 2.1553 Pr = 0.340
Fisher's exact = 0.291

snail1	gender Female	Male	Total
0	39	25	64
	36.5	27.5	64.0
	68.42	58.14	64.00
1	17	18	35
	19.9	15.1	35.0
	29.82	41.86	35.00
0	1	0	1
	0.6	0.4	1.0
	1.75	0.00	1.00
Total	57	43	100
	57.0	43.0	100.0
	100.00	100.00	100.00

Pearson chi2(2) = 2.1737 Pr = 0.337
Fisher's exact = 0.337

(III) Vimentin:

vimentin	stage I	II	III	IV	Total
0	32	13	44	9	98
	32.3	13.7	43.1	8.8	98.0
	96.97	92.86	100.00	100.00	98.00
1	1	1	0	0	2
	0.7	0.3	0.9	0.2	2.0
	3.03	7.14	0.00	0.00	2.00
Total	33	14	44	9	100
	33.0	14.0	44.0	9.0	100.0
	100.00	100.00	100.00	100.00	100.00

Pearson chi2(3) = 3.1496 Pr = 0.369
Fisher's exact = 0.284

vimentin	grade 1	2	3	Total
0	22	67	9	98
	21.6	66.6	9.8	98.0
	100.00	98.53	90.00	98.00
1	0	1	1	2
	0.4	1.4	0.2	2.0
	0.00	1.47	10.00	2.00
Total	22	68	10	100
	22.0	68.0	10.0	100.0
	100.00	100.00	100.00	100.00

Pearson chi2(2) = 3.8115 Pr = 0.149
Fisher's exact = 0.238

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vimentin	lymphnodemetastasis No	Yes	Total
0	62	36	98
	62.7	35.3	98.0
	96.88	100.00	98.00
1	2	0	2
	1.3	0.7	2.0
	3.13	0.00	2.00
Total	64	36	100
	64.0	36.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 1.1480 Pr = 0.284
Fisher's exact = 0.535
1-sided Fisher's exact = 0.407

vimentin	siteofcolon Left	Right	Total
0	55	43	98
	54.9	43.1	98.0
	98.21	97.73	98.00
1	1	1	2
	1.1	0.9	2.0
	1.79	2.27	2.00
Total	56	44	100
	56.0	44.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 0.0298 Pr = 0.863
Fisher's exact = 1.000
1-sided Fisher's exact = 0.689

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vimentin	gender Female	Male	Total
0	55	43	98
	55.9	42.1	98.0
	96.49	100.00	98.00
1	2	0	2
	1.1	0.9	2.0
	3.51	0.00	2.00
Total	57	43	100
	57.0	43.0	100.0
	100.00	100.00	100.00

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          Pearson chi2(1) =   1.5396   Pr = 0.215
           Fisher's exact =                 0.505
   1-sided Fisher's exact =                 0.322

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(IV) N-cadherin:

ncadherin	stage I	II	III	IV	Total
0	33	14	44	7	98
	32.3	13.7	43.1	8.8	98.0
	100.00	100.00	100.00	77.78	98.00
1	0	0	0	2	2
	0.7	0.3	0.9	0.2	2.0
	0.00	0.00	0.00	22.22	2.00
Total	33	14	44	9	100
	33.0	14.0	44.0	9.0	100.0
	100.00	100.00	100.00	100.00	100.00

Pearson chi2(3) = 20.6349 Pr = 0.000
Fisher's exact = 0.007

ncadherin	grade 1	2	3	Total
0	22	66	10	98
	21.6	66.6	9.8	98.0
	100.00	97.06	100.00	98.00
1	0	2	0	2
	0.4	1.4	0.2	2.0
	0.00	2.94	0.00	2.00
Total	22	68	10	100
	22.0	68.0	10.0	100.0
	100.00	100.00	100.00	100.00

Pearson chi2(2) = 0.9604 Pr = 0.619
Fisher's exact = 1.000

ncadherin	lymphnodemetastasis No	Yes	Total
0	64	34	98
	62.7	35.3	98.0
	100.00	94.44	98.00
1	0	2	2
	1.3	0.7	2.0
	0.00	5.56	2.00
Total	64	36	100
	64.0	36.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 3.6281 Pr = 0.057
Fisher's exact = 0.127
1-sided Fisher's exact = 0.127

ncadherin	siteofcolon Left	Right	Total
0	55	43	98
	54.9	43.1	98.0
	98.21	97.73	98.00
1	1	1	2
	1.1	0.9	2.0
	1.79	2.27	2.00
Total	56	44	100
	56.0	44.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 0.0298 Pr = 0.863
Fisher's exact = 1.000
1-sided Fisher's exact = 0.689

ncadherin	gender Female	Male	Total
0	55	43	98
	55.9	42.1	98.0
	96.49	100.00	98.00
1	2	0	2
	1.1	0.9	2.0
	3.51	0.00	2.00
Total	57	43	100
	57.0	43.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 1.5396 Pr = 0.215
Fisher's exact = 0.505
1-sided Fisher's exact = 0.322

(V) CD44v6:

cd44v6	stage I	II	III	IV	Total
0	27	10	29	4	70
	23.1	9.8	30.8	6.3	70.0
	81.82	71.43	65.91	44.44	70.00
1	6	4	15	5	30
	9.9	4.2	13.2	2.7	30.0
	18.18	28.57	34.09	55.56	30.00
Total	33	14	44	9	100
	33.0	14.0	44.0	9.0	100.0
	100.00	100.00	100.00	100.00	100.00

Pearson chi2(3) = 5.3580 Pr = 0.147
Fisher's exact = 0.150

cd44v6	grade 1	2	3	Total
0	16	47	7	70
	15.4	47.6	7.0	70.0
	72.73	69.12	70.00	70.00
1	6	21	3	30
	6.6	20.4	3.0	30.0
	27.27	30.88	30.00	30.00
Total	22	68	10	100
	22.0	68.0	10.0	100.0
	100.00	100.00	100.00	100.00

Pearson chi2(2) = 0.1031 Pr = 0.950
Fisher's exact = 1.000

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cd44v6	lymphnodemetastasis No	Yes	Total
0	48	22	70
	44.8	25.2	70.0
	75.00	61.11	70.00
1	16	14	30
	19.2	10.8	30.0
	25.00	38.89	30.00
Total	64	36	100
	64.0	36.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 2.1164 Pr = 0.146
Fisher's exact = 0.175
1-sided Fisher's exact = 0.110

cd44v6	siteofcolon Left	Right	Total
0	41	29	70
	39.2	30.8	70.0
	73.21	65.91	70.00
1	15	15	30
	16.8	13.2	30.0
	26.79	34.09	30.00
Total	56	44	100
	56.0	44.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 0.6262 Pr = 0.429
Fisher's exact = 0.511
1-sided Fisher's exact = 0.283

cd44v6	gender Female	Male	Total
0	41	29	70
	39.9	30.1	70.0
	71.93	67.44	70.00
1	16	14	30
	17.1	12.9	30.0
	28.07	32.56	30.00
Total	57	43	100
	57.0	43.0	100.0
	100.00	100.00	100.00

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          Pearson chi2(1) =   0.2351   Pr = 0.628
           Fisher's exact =                 0.664
   1-sided Fisher's exact =                 0.394

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Appendix M: Relationship between E-cadherin and mesenchymal/stem cell markers (Stata 12 data)

(I) E-cadherin vs N-cadherin:

ecadherin	ncadherin 0	1	Total
0	76	1	77
	75.5	1.5	77.0
	77.55	50.00	77.00
1	22	1	23
	22.5	0.5	23.0
	22.45	50.00	23.00
Total	98	2	100
	98.0	2.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 0.8401 Pr = 0.359
Fisher's exact = 0.409
1-sided Fisher's exact = 0.409

(II) E-cadherin vs Snail-1:

ecadherin	snail1 0	1	0	Total
0	53	23	1	77
	49.3	26.9	0.8	77.0
	82.81	65.71	100.00	77.00
1	11	12	0	23
	14.7	8.1	0.2	23.0
	17.19	34.29	0.00	23.00
Total	64	35	1	100
	64.0	35.0	1.0	100.0
	100.00	100.00	100.00	100.00

Pearson chi2(2) = 4.0368 Pr = 0.133
Fisher's exact = 0.134

(III) E-cadherin vs Vimentin:

ecadherin	vimentin 0	1	Total
0	75	2	77
	75.5	1.5	77.0
	76.53	100.00	77.00
1	23	0	23
	22.5	0.5	23.0
	23.47	0.00	23.00
Total	98	2	100
	98.0	2.0	100.0
	100.00	100.00	100.00

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          Pearson chi2(1) =   0.6096   Pr = 0.435
           Fisher's exact =                 1.000
   1-sided Fisher's exact =                 0.591

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(IV) E-cadherin vs CD44v6

ecadherin	cd44v6 0	1	Total
0	55	22	77
	53.9	23.1	77.0
	78.57	73.33	77.00
1	15	8	23
	16.1	6.9	23.0
	21.43	26.67	23.00
Total	70	30	100
	70.0	30.0	100.0
	100.00	100.00	100.00

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          Pearson chi2(1) =   0.3253   Pr = 0.568
           Fisher's exact =                 0.609
   1-sided Fisher's exact =                 0.372
```

Appendix N: Relationship between CD44v6 and EMT markers (Stata 12 data)

(I) CD44v6 vs E-cadherin:

cd44v6	ecadherin 0	1	Total
0	55	15	70
	53.9	16.1	70.0
	71.43	65.22	70.00
1	22	8	30
	23.1	6.9	30.0
	28.57	34.78	30.00
Total	77	23	100
	77.0	23.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 0.3253 Pr = 0.568
Fisher's exact = 0.609
1-sided Fisher's exact = 0.372

(II) CD44v6 vs N-cadherin

cd44v6	ncadherin 0	1	Total
0	69	1	70
	68.6	1.4	70.0
	70.41	50.00	70.00
1	29	1	30
	29.4	0.6	30.0
	29.59	50.00	30.00
Total	98	2	100
	98.0	2.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 0.3887 Pr = 0.533
Fisher's exact = 0.512
1-sided Fisher's exact = 0.512

(III) CD44v6 vs Snail-1:

cd44v6	snail1 0	1	0	Total
0	45	25	0	70
	44.8	24.5	0.7	70.0
	70.31	71.43	0.00	70.00
1	19	10	1	30
	19.2	10.5	0.3	30.0
	29.69	28.57	100.00	30.00
Total	64	35	1	100
	64.0	35.0	1.0	100.0
	100.00	100.00	100.00	100.00

Pearson chi2(2) = 2.3703 Pr = 0.306
Fisher's exact = 0.419

(IV) CD44v6 vs Vimentin:

cd44v6	vimentin 0	1	Total
0	69	1	70
	68.6	1.4	70.0
	70.41	50.00	70.00
1	29	1	30
	29.4	0.6	30.0
	29.59	50.00	30.00
Total	98	2	100
	98.0	2.0	100.0
	100.00	100.00	100.00

Pearson chi2(1) = 0.3887 Pr = 0.533
Fisher's exact = 0.512
1-sided Fisher's exact = 0.512

Appendix O: E-cadherin expression in stages 3 and 4 versus other stages

E-cadherin expression: Stage 1 vs Stage 4

stage1	stage4 0	1	Total
0	19	5	24
	20.4	3.6	24.0
	67.86	100.00	72.73
1	9	0	9
	7.6	1.4	9.0
	32.14	0.00	27.27
Total	28	5	33
	28.0	5.0	33.0
	100.00	100.00	100.00

Pearson chi2(1) = 2.2098 Pr = 0.137
Fisher's exact = 0.290
1-sided Fisher's exact = 0.179

E-cadherin expression: Stage 2 vs Stage 4

stage2	stage4 0	1	Total
0	10	1	11
	10.2	0.8	11.0
	76.92	100.00	78.57
1	3	0	3
	2.8	0.2	3.0
	23.08	0.00	21.43
Total	13	1	14
	13.0	1.0	14.0
	100.00	100.00	100.00

Pearson chi2(1) = 0.2937 Pr = 0.588
Fisher's exact = 1.000
1-sided Fisher's exact = 0.786

E-cadherin expression: Stage 3 vs Stage 4

stage3	stage4 0	1	Total
0	27	9	36
	26.0	10.0	36.0
	87.10	75.00	83.72
1	4	3	7
	5.0	2.0	7.0
	12.90	25.00	16.28
Total	31	12	43
	31.0	12.0	43.0
	100.00	100.00	100.00

Pearson chi2(1) = 0.9289 Pr = 0.335
Fisher's exact = 0.378
1-sided Fisher's exact = 0.296

E-cadherin expression: Stage 1 vs Stage 3

stage1	stage3 0	1	Total
0	21	3	24
	21.1	2.9	24.0
	72.41	75.00	72.73
1	8	1	9
	7.9	1.1	9.0
	27.59	25.00	27.27
Total	29	4	33
	29.0	4.0	33.0
	100.00	100.00	100.00

Pearson chi2(1) = 0.0119 Pr = 0.913
Fisher's exact = 1.000
1-sided Fisher's exact = 0.705

E-cadherin expression: Stage 2 vs Stage 3

Stage2	Stage3 0	Total
0	9	9
	9.0	9.0
	75.00	75.00
1	3	3
	3.0	3.0
	25.00	25.00
Total	12	12
	12.0	12.0
	100.00	100.00

Conference proceedings

1. ***Anelisa Jaca, Richard Naidoo and Michael Locketz. National Health Laboratory Service (NHLS) Research Day (2014), Groote Schuur Hospital, Cape Town.*** *Investigating the relationship between miRNA expression and Epithelial Mesenchymal Transition (EMT) in colorectal cancer.*

2. ***Anelisa Jaca, Richard Naidoo and Michael Locketz. University of Cape Town Research Day (2015).*** *The role of miRNA-21 expression and Epithelial Mesenchymal Transition (EMT) in colorectal cancer.*

3. ***Anelisa Jaca, Richard Naidoo and Michael Locketz. National Health Laboratory Service (NHLS) Research Day (2015), Groote Schuur Hospital.*** *The role of miRNA-21 expression and Epithelial Mesenchymal Transition (EMT) in colorectal cancer.*