Molecular analysis of genes involved in iron overload implicated in oesophageal cancer

Veronique Human

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:……

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Summary

Oesophageal cancer is a disease characterised by a disproportionate presentation in certain ethnic groups, with squamous cell carcinoma (SCC) occuring more often in Blacks and adenocarcinoma (ADC) being more prevalent in Caucasians. Several factors have been attributed to the development of OC, including an excess of iron (leading to enhanced tumour growth), oesophageal injury and chronic inflammation.

The main aim of this study was to establish the mutation spectrum of six genes (including *HFE*, *HMOX1*, *SLC40A1, HAMP*, *CYBRD1* and *HJV*) involved in iron metabolism, in the Black South African OC population. The patient cohort comprised of 50 (25 male and 25 female) unrelated patients presenting with SCC of the oesophagus, with the control group consisting of 50 unrelated, healthy population-matched individuals. The mutation detection techniques employed included polymerase chain reaction (PCR) amplification, heteroduplex single-stranded conformational polymorphism (HEX-SSCP) analysis, restriction fragment length polymorphism (RFLP) analysis and bi-directional semi-automated DNA sequencing analysis of variants identified.

Twenty-one previously described and thirteen novel variants (***HFE***: Y342; ***HMOX1***: G255R, R262H, R262C; ***SLC40A1***: IVS5-27A→C, L378, 3'UTR+284C→T, 3'UTR+289G→A, 3'UTR+289G→T; ***CYBRD1***: L17, P195; ***HJV***: 5'UTR-1401T→C, 3'UTR+47A→G) were identified in this study. No statistically significant associations were observed for the variants identified.

Oesophageal cancer is insidious in onset, because symptoms present late in the development process. After diagnosis of these symptoms, treatment is highly ineffective. The only hope for effective intervention is early detection and subsequent treatment. This can only be achieved by the establishment of an effective screening programme in high incidence areas. This is the first study signifying the potential contribution of iron dysregulation to OC susceptibility in the Black South African population, thus possibly setting the foundation for the aforementioned screening programme.

Opsomming

Oesofageale kanker (OK) is ‘n siekte wat gekenmerk word deur ‘n disproporsionele verteenwoordiging in sekere etniese groepe, met plaveisel-selkarsinoom (SSC) wat meer gereeld voorkom in Swart populasies en adenokarsinoom (ADC) wat oorwegend in Kaukasiërs voorkom. ‘n Aantal faktore is al toegeskryf aan die ontwikkeling van OK, insluitend ‘n oormaat yster (wat lei tot verhoogde gewasgroei), oesofageale besering en kroniese ontsteking.

Die hoofdoel van hierdie studie was die bepaling van die mutasie spektrum van ses gene (insluitend *HFE*, *HMOX1*, *SLC40A1*, *HAMP*, *CYBRD1* en *HJV*) betrokke by ystermetabolisme, in die Swart Suid-Afrikaanse OK populasie. Die pasiënt groep het bestaan uit 50 (25 manlike en 25 vroulike) onverwante pasiënte by wie SCC van die oesofagus voorgekom het, terwyl die kontrole groep bestaan het uit 50 onverwante, gesonde bevolkings-gelyke individue. Die mutasie opsporingstegnieke wat gebruik is, het polimerase kettingreaksie (PKR) amplifisering, heterodupleks enkelstring konformasie polimorfisme (HEX-SSCP) analise, restriksie fragment lengte polimorfisme (RFLP) analise en tweerigting semi-geoutomatiseerde DNS volgorde-bepalingsanalise van die geïdentifiseerde variante ingesluit.

Een-en-twintig reeds beskryfde en dertien nuwe variante (***HFE***: Y342; ***HMOX1***: G255R, R262H, R262C; ***SLC40A1***: IVS5-27A→C, L378, 3’UTR+284C→T, 3’UTR+289G→A, 3’UTR+289G→T; ***CYBRD1***: L17, P195; ***HJV***: 5’UTR-1401T→C, 3’UTR+47A→G) is in hierdie studie geïdentifiseer. Geen statisties betekenisvolle assosiasie met die geïdentifiseerde variante is waargeneem nie.

Oesofageale kanker is gevaarlik in aanvangs, omdat die simptome laat in die ontwikkelingsproses uitgebeeld word. Na diagnose van hierdie simptome, is behandeling hoogs oneffektief. Die enigste hoop vir effektiewe ingryping is vroegtydige opsporing en die gevolglike behandeling, wat slegs bereik word deur die opstelling van ‘n doeltreffende siftingsprogram in hoë risiko areas. Hierdie is die eerste studie wat die moontlike bydrae van yster disregulasie tot OK vatbaarheid in die Swart Suid-Afrikaanse populasie aandui, en sodoende, bied dit moontlik die grondslag vir die voorafgenoemde siftingsprogram.

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'What lies behind us and what lies before us are tiny matters compared to what lies within us.'

Oliver Wendell Holmes

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List of Abbreviations and Symbols

5’	5-prime
3’	3-prime
α	alpha
β	beta
χ^2	chi-squared
ºC	degrees Celsius
=	equal to
>	larger than
µg/l	microgram per litre
µl	microlitre
-	minus
%	percentage
+	plus
±	plus-minus
®	registered trademark
<	smaller than
x g	times gravity

A	adenine
A	alanine
AA	acrylamide
ABC7	adenosine triphosphate-binding cassette transporter 7
ACD	anaemia of chronic disease
ADC	adenocarcinoma of the oesophagus
ADHs	alcohol dehydrogenases
$AgNO_3$	silver nitrate
ALDs	aldehyde dehydrogenases
APC	adenomatous polyposis of the colon gene
APS	ammonium persulphate
ASIR	age standardised incidence rate
ASSP	alternative splice site predictor

ATP	adenosine 5’-triphosphate
BAA	bisacrylamide
bp	base pair
BLAST	basic local alignment search tool
BMI	body mass index
C	crosslinking
C	cysteine
C	cytosine
CCND1	cyclin D1 gene
cDNA	complementary deoxyribonucleic acid
CGH	comparative genomic hybridisation
$C_{19}H_{10}Br_4O_5S$	bromophenol blue
$CH_3(CH_2)_{11}OSO_3Na$	sodium dodecyl sulphate
CH_3COOH	acetic acid
$C_{31}H_{28}N_2Na_4O_{13}S$	xylene cyanol
$C_4H_{11}NO_3$	tris-HCl
cm	centimetre
C-MYC	c-myc gene
CO_2	carbon dioxide
COX1	cyclooxygenase-1 protein
CP	ceruloplasmin gene
CYBRD1	cytochrome b reductase 1 gene
CYPs	cytochrome P450 genes
D	aspartic acid
dATP	2’-deoxy-adenosine-5’-triphosphate
DCC	deleted in colorectal carcinoma gene
dCTP	2’-deoxy-guanosine-5’-triphosphate
DCT1	divalent cation transporter-1 gene
DCYTB	duodenal cytochrome b gene
del	deletion

dGTP	2’-deoxy-guanosine-5’-triphosphate
dHPLC	denaturing high performance liquid chromatography
DMT1	divalent metal transporter 1 gene
DNA	deoxyribonucleic acid
dNTP	2’-deoxy-nucleotide-5’-triphosphate
dTTP	2’-deoxy-thymidine-5’-triphosphate
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor gene
ESE(s)	exonic splicing enhancer(s)
EST	expressed sequence tag
EtBr	ethidium bromide
EtOH	ethanol
F	forward primer
FBN-1	fibrillin-1 gene
Fe	iron
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
FEZ1	F37/Oesophageal cancer-related gene-coding leucine-zipper motif gene
FHIT	fragile histidine triad gene
FISH	fluorescent in situ hybridisation
FPN1	ferroportin 1 gene
g	gram
G	glycine
G	guanine
GORD	gastro-oesophageal reflux disease
GPI	glycosyl-phosphatidylinositol
GSTs	glutathione S-transferases
GSTM1	glutathione S-transferases M1 gene
GSTP1	glutathione S-transferases P1 gene

GSTT1	glutathione S-transferases T1 gene
H	histidine
Hamp	hepcidin antimicrobial peptide mouse gene
HAMP	hepcidin antimicrobial peptide gene
H_3BO_3	boric acid
HCHO	formaldehyde
HEPC	hepcidin gene
HEPH	hephaestin gene
HEX-SSCP	heteroduplex single-strand conformation polymorphism analysis
Hfe	high iron mouse gene
HFE	high iron gene
HH	hereditary haemochromatosis
Hjv	hemojuvelin mouse gene
HJV	hemojuvelin gene
HLA-A2	major histocompatibility complex class I A2
HLA-G1	major histocompatibility complex class I G1
Hmox1	haem oxygenase 1 mouse gene
HMOX1	haem oxygenase 1 gene
H_2NCHO	formamide
H_2O_2	hydrogen peroxide
HPV	human papilloma virus
HWE	Hardy-Weinberg equilibrium
I	isoleucine
IFN-γ	interferon-gamma
iNOS	inducible nitric oxide synthase protein
IRE(s)	iron response element(s)
IREG1	iron-regulated transporter 1 gene
IRP	iron responsive protein
IRP-1	iron regulatory protein 1
IRP-2	iron regulatory protein 2

IVS	intervening sequence
JH	juvenile haemochromatosis
KCl	potassium chloride
kDa	kilo Dalton
$KHCO_3$	potassium hydrogen carbonate
KH_2PO_4	potassium dihydrogen orthophosphate
L	leucine
LEAP1	liver-expressed antimicrobial peptide 1 gene
LOH	loss of heterozygosity
M	methionine
M	molar
MCC	mutated in colorectal cancers gene
MDM2	mouse double minute 2 homolog gene
MFS	Marfan syndrome
mg	milligram
$MgCl_2$	magnesium chloride
mg/ml	milligram per millilitre
MHC	major histocompatibility complex
min	minutes
ml	millilitre
mm	millimetre
mM	milli-molar
mRNA	messenger ribonucleic acid
MTP1	metal transporter 1 gene
N	asparagine
NaCl	sodium chloride
NaOH	sodium hydroxide

NAT-2	N-acetyltransferase 2 gene
ng	nanogram
ng/µl	nanogram per microlitre
NH_4Cl	ammonium chloride
$(NH_2)_2CO$	urea
$(NH_4)_2S_2O_8$	ammonium persulphate
NRAMP1	natural resistance-associated macrophage protein 1 gene
NRAMP2	natural resistance-associated macrophage protein 2 gene
NTBI	non-transferrin-bound iron
O_2^-	superoxide
OC	oesophageal cancer
OH	hydroxyl
OMIM	Online Mendelian Inheritance in Man
p	short arm of chromosome
P	probability
P	proline
PAA	polyacrylamide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	potential of hydrogen
PKU	phenylketonuria
pmol	picomole
Pvu II	*Proteus vulgaris*, 2nd enzyme
q	long arm of chromosome
Q	glutamine
R	arginine
R	reverse primer
RACE	rapid amplification of cDNA ends
RB1	retinoblastoma gene

RBC	red blood cell
RE	reticoendothelial
RFLP	restriction fragment length polymorphism
RGD	arginine-glycine-aspartic acid motif
RGM	repulsive guidance molecule
RNA	ribonucleic acid
ROS	reactive oxygen species
RsaI	*Rhodopseudomonas sphaeroides*, 1st enzyme
RT-PCR	reverse transcriptase polymerase chain reaction
S	serine
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate
SFT	stimulator of (Fe) iron transport
SLC11A2	solute carrier family 11 (proton-coupled divalent metal ion transporter) number 2 gene
SLC11A3	solute carrier family 11 (proton-coupled divalent metal ion transporter) number 3 gene
Slc40a1	solute carrier family 40 (iron regulated transporter) member 1 mouse gene
SLC40A1	solute carrier family 40 (iron regulated transporter) member 1 gene
SNP(s)	single nucleotide polymorphism(s)
SR-proteins	serine-arginine rich proteins
SSCP	single-strand conformational polymorphisms
T	threonine
T	thymine
T_A	annealing temperature
Taq	*Thermus aquaticus* enzyme
TBE	Tris-Borate-EDTA buffer
TBI	transferrin-bound iron
TEMED	N', N, N', N'-tetramethylethylenediamine

Tf	transferrin
TFR1	transferrin receptor 1 gene
TFR2	transferrin receptor 2 gene
TGFR	transforming growth factor receptor
T_M	melting temperature
TP53	tumour protein p53
Tris-HCl	tris hydrochloride [2-Amino-2-(hydroxymethyl)-1,3 propanediol - hydrochloride]
TS	transferrin saturation
U	units
UK	United Kingdom
USA	United States of America
UTR	untranslated region
Usf2	upstream stimulatory factor 2 mouse gene
V	valine
V	volts
v/v	volume per volume
w/v	weight per volume
x	times
X	X-chromosome
XRCC1	X-ray repair cross complementing 1 gene
Y	tyrosine

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Chapter 1

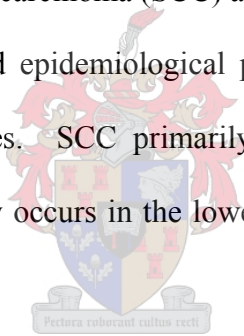
Literature Review

1. Literature Review

1.1. Oesophageal Cancer (OC)

1.1.1 Disease definition and classification

OC is a malignancy that develops in tissue lining the hollow, muscular canal along which food and liquids travel from the throat to the stomach. It originates in the inner layers of the lining of the oesophagus, growing outward. This disease can be classified into two major histological subtypes: squamous cell carcinoma (SCC) and adenocarcinoma (ADC). SCC and ADC have dissimilar biological and epidemiological profiles and subsequently, should be regarded as separate disease entities. SCC primarily occurs in the middle third of the oesophagus and ADC predominantly occurs in the lower third of the oesophagus (Yang and Davis 1988).



The less common histological types include adenoid cystic cancer, adenosquamous cancer, as well as primary malignant melanoma, mucoepidermoid and undifferentiated cancer (Koshy et al. 2004). OC is known for its aggressive clinical behaviour and poor prognosis. It develops in mid to late adulthood and is rarely seen in individuals younger than 25. The mortality rates show a steady increase with age (Blot 1994).

1.1.2 Presentation and progression

The majority of OC patients present with symptoms such as dysphagia and extreme weight loss. Weight loss can be considered an independent indicator of poor prognosis, if a loss of more than 10% is detected in a patient's body mass (Fein et al. 1985). The less common symptoms of OC are odynophagia (pain in swallowing foods and liquids), hoarseness, melena, cachexia and retrosternal pain (Ojala et al. 1982). Constant heartburn, nausea and vomiting should also be considered indicators of oesophageal cancer.

OC tends to present at an advanced stage. The majority of individuals that have developed OC do not exhibit the symptoms until the tumour is large enough to cause mechanical obstruction. It has been found that more than 50% of patients present with either unresectable tumours or radiographically visible metastases when diagnosed with oesophageal cancer (Enzinger and Mayer 2003). Generally, with the development of cancer, approximately 21% of submucosal cancers and up to 60% of cancers that invade the muscles may be associated with the spreading to the lymph nodes (Siewert et al. 2001, Collard 2001).

SCC spreads in a linear submucosal manner, while ADC spreads by transversely penetrating the oesophageal wall. Autopsy specimens have indicated the erratic manner in which OC spreads through the extensive lymphatic channels (Hosch et al. 2001). ADC develops from gastro-oesophageal reflux disease (GORD) through a sequence of events. This includes the development of inflammation-stimulated hyperplasia and metaplasia, followed by multifocal dysplasia, carcinoma *in situ* and invasive adenocarcinoma.

1.1.3 Demographics and epidemiology of oesophageal cancer

Oesophageal cancer displays a striking geographic variation in incidence, occurring at high frequencies in certain defined global regions. The highest risk areas of the world are the so-called "Asian oesophageal cancer belt" (Eastern Turkey, Iran, Iraq, China, Hong Kong, Japan), France, South and East Africa and eastern South America (Blot 1994, Parkin et al. 2005). In South Africa, the highest OC incidence has been found in the Transkei region.

The incidence of SCC, the most common subtype of oesophageal cancer (Vizcaino et al. 2002), has remained relatively stable over the last few decades, while the incidence of ADC has displayed an increase. A three- to fourfold greater risk exists for males to develop SCC and males have a seven- to tenfold higher risk than females for developing ADC (Pickens and Orringer 2003).

SCC, occurring more frequently in Blacks than Caucasians, is considered the leading cause of cancer death among males of the Black South African population (age standardised incidence rate [ASIR] 13.6/100,000) and the fourth most common cancer among Coloured males of South Africa (Coloured referring to individuals of mixed ancestry; ASIR 7.7/100,000). Among Black females, SCC is the third most common cancer (ASIR 5.8/100,000) (Sitas et al. 1998). ADC is more prevalent in Caucasians (Blot 1994). These two subtypes display distinct aetiological and pathological characteristics (Kuwano et al. 2005). These epidemiological differences could potentially play a vital role in understanding the aetiology and pathogenesis of oesophageal cancer (Pickens and Orringer 2003).

1.1.4 Factors involved in OC pathogenesis

The geographic and ethnic variation in incidence observed in the epidemiology of OC could be due to the degree of exposure to certain environmental factors, the type of exogenous factors and the genetic susceptibility of populations in these regions.

1.1.4.1 Environmental factors

Various environmental factors have been implicated as risk factors of OC. These elements render the oesophageal mucosa more susceptible to carcinogenic injury, subsequently leading to oesophageal cancer.

1.1.4.1.1 Alcohol consumption and tobacco use

In Western countries, alcohol consumption and cigarette smoking are regarded as the main causes of SCC (IARC 1986, IARC 1988). Due to the various carcinogens present in tobacco tar and cigarette smoke, tobacco use has been implicated as a major risk factor for OC (Auerbach et al. 1965). Studies conducted in South Africa found the majority of SCC patients were smokers and consumed alcoholic beverages, specifically home-brewed beer produced from maize (Segal et al. 1988, Sumeruk et al. 1992). The homegrown tobacco used was either hand-rolled into cigarettes or chewed by the patients.

Segal et al. (1988) have shown that in the South African population, 75% of OC patients were smokers and 80% of patients consumed traditional home-brewed and commercial African beer. Cigarette smokers have a fivefold higher risk of developing OC than non-smokers and

the increased consumption of alcohol among smokers further increases the risk of OC, in a synergistic manner (Blot 1994). However, although considered as significant risk factors in countries such as South Africa, tobacco and alcohol consumption are not implicated in the aetiology of OC in other high-risk areas such as China and Iran. This phenomenon suggests the involvement of other major risk factors in OC development.

1.1.4.1.2 Nutrition

Deficiency of various micronutrients such as riboflavin, zinc and iron occur in diets mainly based on cereal. Groenewald et al. (1981) observed that the diets of children and nursing mothers, from areas of moderate to high OC incidence in Transkei, were deficient in these micronutrients.

A common risk factor of OC is the mycotoxin fumonisins, from the fungus *Fusarium verticillioides*, formerly known as *Fusarium moniliforme* (Marasas et al. 1988). These mycotoxins occur on corn that is intended for human consumption. A correlation has been observed between the incidence of fumonisins in home-grown corn with the incidence of OC in the Transkei region (Marasas et al. 1988). It is proposed that the mycotoxins indirectly influence DNA synthesis due to its influence on the normal sphingolipid metabolism in the cell (Seegers et al. 2000). N-nitrosamines (also found in food infected with fungi) have also been found to be carcinogenic for the oesophagus (Preussmann 1984). Pickled vegetables and foods that may become mouldy or fermented have also been associated with an increased risk of OC in a study conducted in Hong Kong (Cheng et al. 1992).

Plummer-Vinson syndrome is a disease related to iron and/or riboflavin deficiencies (Anthony et al. 1999). Patients with Plummer-Vinson syndrome display symptoms that include upper oesophageal strictures, glossitis and dysphagia. A high prevalence of OC has been observed in patients suffering from Plummer-Vinson syndrome.

1.1.4.1.3 Viral pathogenesis

Since 1982, when the human papilloma virus (HPV) was first implicated in the pathogenesis of oesophageal cancer (Syrjanen 1982), the presence of HPV in patients has been tested and confirmed by various methods including immunohistochemistry and polymerase chain reaction (PCR) amplification. In areas of low risk for OC and HPV infection (such as the USA and various European countries) not many cases of HPV were detected, but the virus was frequently identified in OC patients from high-risk countries such as South Africa, Japan and China (Lam 2000, Matsha et al. 2002).

Although HPV is not associated with the occurrence of OC in low risk areas such as the United Kingdom and Italy (Morgan et al. 1997, Rugge et al. 1997), a notably high prevalence (46%) of HPV infections was found in SCC patients from the Transkei region of South Africa (Matsha et al. 2002). The HPV virus is clearly an important factor in the pathogenesis of SCC, but due to the low overall incidence it is suggested that HPV may have a synergistic effect with other risk factors in the pathogenesis of OC (Lam 2000).

1.1.4.1.4 Gastro-oesophageal reflux and Barrett's oesophagus

Gastro-oesophageal reflux is the largest risk factor of adenocarcinoma and is characterised by the movement of the stomach contents into the oesophagus (Largergren et al. 1999). The factors contributing to reflux include oesophageal ulceration, achalasia and hiatus hernia. The development of Barrett's oesophagus accounts for the occurrence of ADC in the lower third of the oesophagus and the gastro-oesophageal junction. Barrett's oesophagus is one of the complications of gastro-oesophageal reflux. The majority of cases are thought to occur sporadically, although a few cases of familial clustering have been observed.

1.1.4.1.5 Other environmental risk factors

Various other risk factors include the consumption of hot beverages (Yang and Wang 1993), a history of oesophageal injury and chronic inflammation, and tannins (Klimstra 1994). Individuals with recurring symptoms of reflux, have an eightfold increased risk of developing ADC (Largergren et al. 1999).

Obesity [body mass index (BMI) > 30] has been identified as a risk factor of ADC, as it has been postulated that obesity can increase the intra-abdominal pressure as well as the occurrence of gastro-oesophageal reflux (Largergren et al. 1999). The prevalence of obesity is increasing in Western populations, which also have the highest incidence of OC (Vaughan et al. 1995).

Another risk factor contributing to OC pathogenesis to a lesser extent is occupational exposure to carcinogens (Selikoff et al. 1979, Norell et al. 1983, Gustavsson et al. 1993,

Parent et al. 2000). Warehouse workers, miners and workers exposed to combustion products are also at an increased risk of developing SCC. Parent et al. (2000) have shown an increased risk of SCC development, if exposed to numerous substances in the workplace, including dust (eg. carbon black), liquids (eg. mineral spirits with benzene and xylene) and vapours (eg. sulphuric acid), metals (iron compounds), aromatic hydrocarbons (eg. polycyclic aromatic hydrocarbons from any source) as well as other substances (eg. formaldehyde).

1.1.4.2 Genetic (genomic and molecular) factors

Various chromosomal abnormalities and gene alterations have been identified in OC. These gene alterations include overexpression and gene inactivation of various genes such as *C-MYC*, epidermal growth factor receptor (*EGFR*, Lu et al. 1988) and cyclin D1 (*CCND1*, Adelaide et al. 1995). The production levels of cyclooxygenase 1 (COX1) and inducible nitric oxide synthase (iNOS) in both mucosal and invasive OC have been found to be elevated in chronically inflamed tissues, including precancerous lesions (Tanaka et al. 1999, Zimmerman et al. 1999).

Only a few comparative genomic hybridisation (CGH) studies on OC have been reported. Several chromosomal regions identified by CGH analysis, have been implicated in OC pathogenesis (Moskaluk et al. 1998, Sarlomo-Rikala et al. 1998, du Plessis et al. 1999). Using CGH, du Plessis et al. (1999) performed a genome-wide screen for the detection of DNA loss and gain in SCC. Frequent loss was identified on chromosomes 1p, 4p, 18q, 19q, and 22q, while DNA gain occurred on chromosomes 1q, 2q, 3q, 5p, 7p, 7q, 8q and Xq. This was the first study providing a record of chromosomal imbalances in OC tumours in the South African Coloured and Black populations.

Loss of heterozygosity (LOH) studies have implicated several chromosomal regions in oesophageal cancer. With the aid of microsatellite markers, LOH had been identified at certain loci on chromosomes 1, 3, 5, 9, 11, 13, 17 and 18 (Huang et al. 1992, Tarmin et al. 1994, Wang et al. 1995, Barrett et al. 1996, Shimada et al. 1996). Frequent chromosomal abnormalities found in OC affecting chromosomes 1, 2, 3, 6, 7, 9 and 11 have also been identified in other studies (Whang-Peng et al. 1990, Rosenblum-Vos et al. 1993, Rao et al. 1995).

Numerous genes involved in cell growth and regulatory pathways have been found altered in OC (reviewed by Klimstra 1994, Rosen 1994, Lam 2000, Mandard et al. 2000, McCabe and Dlamini 2005). Alterations such as allelic loss, frequent mutations and deletions have been identified in the p53 tumour suppressor gene (*TP53*), retinoblastoma (*RB1*), mutated in colorectal cancer (*MCC*), deleted in colorectal carcinoma (*DCC*) and adenomatous polyposis of the colon (A*P*C) genes (reviewed by Lu 2000). In approximately 20% of SCC patients, amplification of *MDM2,* which diminishes the function of normal *TP53*, has been detected (Shibagaki et al. 1995). Tumour suppressor genes that have recently been implicated in SCC pathogenesis include the fragile histidine triad (*FHIT*) and F37/Oesophageal cancer-related gene-coding leucine-zipper motif (*FEZ1*) (Ishii et al. 1999, Menin et al. 2000, Nishiwaki et al. 2000).

Individual susceptibility to cancer may also be influenced by genetic polymorphisms in certain enzymes that are involved in the metabolism of environmental carcinogenesis. These enzymes include the cytochromes P450 (*CYPs*), glutathione S-transferases (GSTs) T1, P1 and M1 (*GSTT1*, *GSTP1*, *GSTM1*), N-acetyltransferase 2 (*NAT-2*), alcohol dehydrogenases (ADHs) and aldehyde dehydrogenases (ALDs). *CYPs* are responsible for the bioactivation of

various low molecular weight carcinogens, *GSTs* are involved in the detoxification of many carcinogenic electrophiles and carcinogens in tobacco smoke, while ADHs and ALDs are alcohol-metabolising enzymes (Hori et al. 1997, Morita et al. 1997, 1998, Nimura et al. 1997, Lin et al. 1998, Van Lieshout et al. 1999, Dandara et al. 2005).

Nonepidermolytic palmoplantar keratoderma, also known as tylosis, is the only recognised familial syndrome that predisposes patients to SCC. Tylosis is a rare autosomal dominant disorder that is defined by a genetic abnormality at chromosomal region 17q25 (Kellsell et al. 1996, Risk et al. 1996, 1999). Various studies have also reported an association of Barrett's oesophagus with ADC displaying a dominant mode of inheritance in several different families (Crabb et al. 1985, Jochem et al. 1992, Eng et al. 1993, Poynton et al. 1996).

Genetic polymorphisms found in the DNA repair genes, may influence the deviation in DNA repair capacity that might be related to an increased risk of cancer development. DNA repair genes that have been implicated in OC susceptibility include the polymorphic X-Ray Repair Cross Complementing 1 (*XRCC1*) genes (Lee et al. 2001).

Due to its roles in iron transport and inflammation, the natural resistance-associated macrophage protein-1 (*NRAMP1*) gene was investigated in the development of OC in the South African population. Significant association have been observed between variation in *NRAMP1* and OC in the Black (du Plessis 2000, Zaahl 2003) and Coloured (Zaahl 2003, Zaahl et al. 2005) South African populations. Collectively all these studies provide evidence that multiple factors at the molecular level are involved in the initiation and development of oesophageal cancer.

1.2. Iron and OC

1.2.1. Proposed mechanisms of iron carcinogenesis

Various *in vitro* and *in vivo* studies have revealed that both conditions of iron-deficiency and iron-overload can be pathogenic. Three mechanisms exist whereby iron can initiate and promote the process of carcinogenesis: 1) the production of hydroxyl radicals and oxidative stress, 2) favouring or promoting the growth of tumour cells and 3) modifying the immune system by suppressing the activity of the defence cells (Toyokuni 1999).

The production of hydroxyl radicals and oxidative stress

The toxicity of iron is based primarily on the Fenton and the Haber-Weiss chemistry. In these reactions, catalytic amounts of iron can yield hydroxyl radicals (OH) from superoxide (O_2^-) and hydrogen peroxide (H_2O_2), collectively termed reactive oxygen species (ROS) (Papanikolaou and Pantopoulos 2005). These free radicals are highly reactive and may participate in the oxidation of proteins, membrane lipid peroxidation as well as the modification of nucleic acids. A surplus of redox-reactive iron may intensify the oxidative stress of the cell, leading to accelerated tissue degeneration. The cell's protective mechanisms against this oxidative stress are mostly reducing agents and enzymes that are associated with reductants. Transferrin is the plasma iron carrier that maintains extracellular iron in a soluble and non-toxic form, under normal physiological conditions. The "free" or unbound iron becomes harmful, as it may produce reducing agents that can initiate the Fenton reaction and subsequently contribute to cancer development (Tokoyuni 1996, Ponka et al. 1998).

Favouring or promoting the growth of tumour cells

An elevated iron supply is required for the sustained proliferation of tumour cells. Tumour cells grow and survive better *in vitro* in the presence of high levels of extracellular iron. Iron supplementation has enhanced the growth of human hepatoma cells (Hann et al. 1990). In hepatocellular carcinoma related to hepatitis B virus infections, iron may have a promoting effect through two possible mechanisms: 1) by facilitating the growth of cancer cells and 2) facilitating the replication of the hepatitis B virus (Zhou et al. 1987).

Modifying the immune system by suppressing the activity of the defence cells

The impairment of macrophage cytotoxic activity against tumour growth is another mechanism of carcinogenesis. In normal anti-tumour processes, iron loss occurs from target cells. Therefore, in conditions of iron overload, the cytotoxic activity of the macrophages is impaired and tumour growth is favoured (Green et al. 1988, Huot et al. 1990). The tumouricidal activity of mice macrophages was markedly decreased by the presence of iron salts, iron-containing ferritin and iron-dextran (Green et al. 1988). Iron also reduces the gamma-interferon activity of macrophages, as iron (or and excess thereof) reduces the activity of interferon-gamma (IFN-γ) and thereby interferes with the growth of the tumouricidal-activated macrophages. This subsequently leads to enhanced tumour growth (Weiss et al. 1992). Iron is also responsible for preventing macrophages from producing the cytotoxic free radical, nitric oxide, subsequently down-regulating the anti-tumour activity of macrophages (Harhaji et al. 2004)

1.2.2. Iron as a risk factor for OC

Previously it had been believed that iron overload associated with OC in the Black South African population resulted from the excessive consumption of home-brewed alcoholic beverages from maize and sorghum beer that were contaminated with iron (MacPhail et al. 1979, Isaacson et al. 1985). In Saudi Arabia, water with high iron content was also associated with an increased risk of developing OC (Amer et al. 1990). An excessive dietary iron intake had been linked to the pathogenesis of hepatocellular carcinoma in the Black population of South Africa, the first study highlighting the role of iron in cancer in this population (Mandishona et al. 1998). Iron overload has also been reported as a risk factor for OC in other populations, including a Danish population with primary haemochromatosis, where an increased risk of OC was illustrated (Hsing et al. 1995).

The pathogenesis of Barrett's oesophagus and its progression to oesophageal adenocarcinoma was studied using a rat model. The iron-supplemented rats of this study had significantly high levels of inflammation, cell proliferation, inducible nitric oxide synthase (iNOS) and nitrotyrosine. These rats also had more tumours in their distal oesophagus than rats receiving no iron supplement (Goldstein et al. 1998). It can thus be concluded that iron supplementation enhanced inflammation, as well as the production of reactive oxygen and nitrogen species in the oesophagus. A progression in epithelial cell proliferation and inflammation was observed, from a mild to severe state in the distal oesophagus of the rats. These processes may contribute to the development of Barrett's oesophagus and subsequently its progression to ADC (Goldstein et al. 1998, Chen et al. 1999, 2000).

The *NRAMP1* gene was examined as a possible OC susceptibility gene in two distinct South African populations. Significant association has been observed between variation in *NRAMP1* and OC in the Black and Coloured South African populations (du Plessis 2000, Zaahl 2003, Zaahl et al. 2005).

1.3. Iron homeostasis

1.3.1. Iron distribution and circulation

Approximately 70% of the body iron is used within haemoglobin that is found in circulating erythrocytes. Iron is transported through the plasma as a complex formed with transferrin, an 80 kDa protein with two iron binding sites (Emerit et al. 2001). Another 20 to 30% of the body iron is stored inside ferritin. It is found within the hepatocytes and the reticoendothelial macrophages, while the remainder of the body iron may be found within the myoglobin, cytochromes and the iron-containing enzymes. The plasma delivers approximately 30 mg of iron to the cells each day (Emerit et al. 2001). The 30 mg of iron required daily for erythropoiesis is provided from macrophage iron recycling. The non-haem iron in the circulation is found bound to transferrin (Andrews 1999).

1.3.2. Intestinal iron absorption

Various proteins that are involved in intestinal iron absorption, as well as the chromosomal locations, are indicated in Table 1.1. The absorption of dietary iron is a multi-step process that requires the uptake of iron from the intestinal lumen, its transfer across the apical cell surface of the villus enterocytes and its subsequent transfer across the basolateral membrane

Table 1.1. Proteins involved in iron metabolism

Protein	Chromosomal location	Function
Transferrin (Tf)	3q21	Acts as an iron-binding transport protein in both the plasma and extracellular fluid
Transferrin receptor 1 (TFR1)	3q29	Principal molecule responsible for the uptake of transferrin bound iron into cells
Transferrin receptor 2	7q22	The precise function is unknown, but is thought to play a role in maintaining iron homeostasis
Ferritin	11q12; 19q13	Involved in iron storage. The H-subunit displays ferroxidase activity, while the L-subunit is responsible for catalysing iron core formation
Iron-regulatory protein 1 (IRP-1) Iron-regulatory protein 2 (IRP-2)	9 15	Involved in translational regulation in the synthesis of the transferrin receptor, ferritin, and other iron-related proteins
High iron protein (HFE)	6p21	HFE protein-β2-microglobulin heterodimer binds TFR, reducing its affinity for transferrin,thus reducing iron uptake
Divalent metal transporter 1 (DMT1)	12q13	The transport of iron: 1) from gastrointestinal lumen into duodenal enterocyte, 2) from erythroblast endosome to the cytoplasm
Haem oxygenase 1 (HMOX1)	22q12	Catalytic oxidation of haem to Fe^{2+}, carbon monoxide and biliverdin
Fe-ATPase iron transporter	unknown	Intracellular transmembrane iron transport in the macrophages; coupled with HMOX1
Solute carrier family 40 (iron-regulated transporter) member 1 (SLC40A1)	2	The transport of iron: at basolateral membrane of the duodenal enterocyte, in the macrophage cytoplasm and in the hepatocyte sinusoidal membrane
Ceruloplasmin (CP)	3q21-24	Serum ferroxidase
Hephaestin (HEPH)	Xq11-12	Possible intracellular ferroxidase
Stimulator of iron transport (SFT)	10q21	Increases cellular uptake of both transferrin-bound and nontransferrin-bound iron
Frataxin	9q13	Mitochondrial iron homeostasis and respiratory function
ATP-binding cassette 7 (ABC7)	Xq13	Possibly involved in mitochondrial iron export
Cytochrome b reductase 1 (CYBRD1)	2	Ferrireductase facilitating enzymatic reduction of inorganic iron

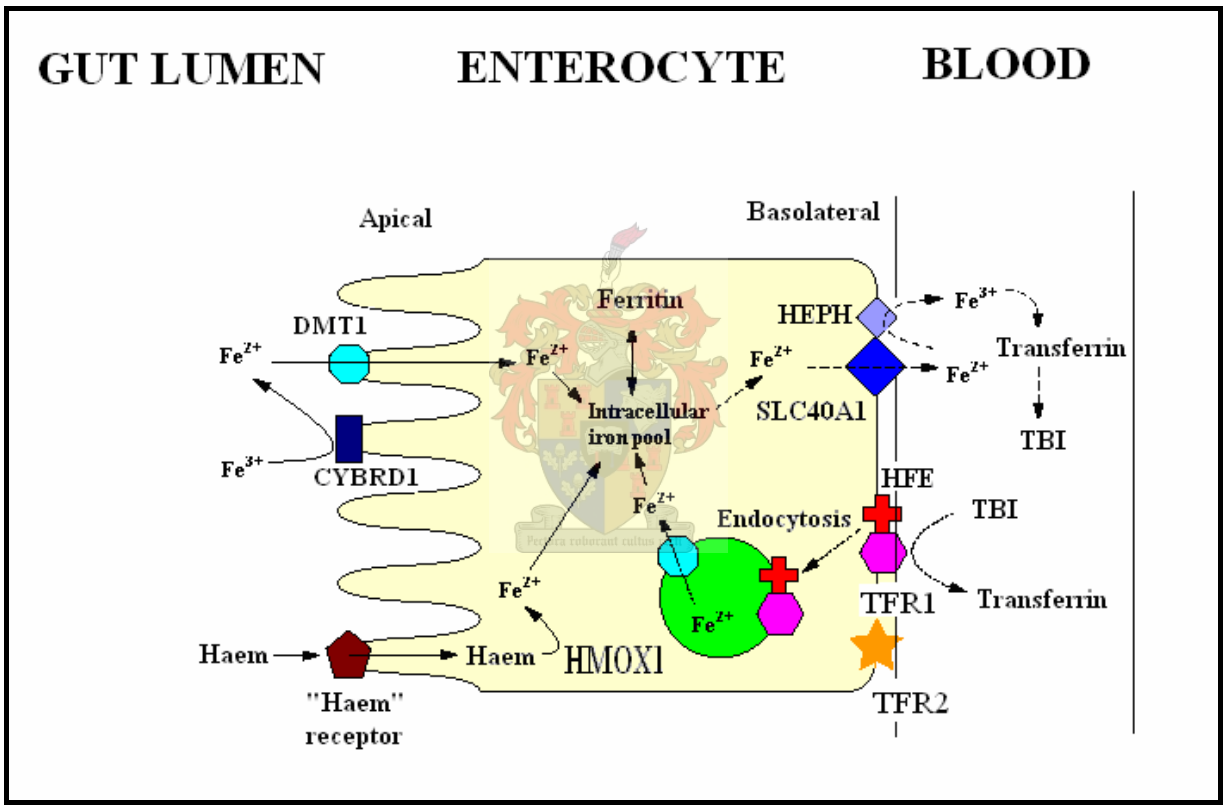
Adapted from Brittenham et al. (2000) and Sheth and Brittenham (2000).

to the plasma (Trinder et al. 2002b). The ingested inorganic iron exists in the oxidised ferric (Fe^{3+}) form and before the absorption of iron can occur, it should first be reduced to its ferrous (Fe^{2+}) form. The low pH of the gastric efflux aids in the absorption of dietary iron by providing a proton rich environment facilitating the enzymatic reduction of the ingested inorganic iron by the brushborder ferrireductase, cytochrome b reductase 1 [(CYBRD1), also known as duodenal cytochrome b (DCYTB), McKie et al. 2001]. The divalent metal transporter 1 [DMT1, also known as divalent cation transporter-1 (DCT1) or natural resistance-associated macrophage protein 2 (NRAMP2)] is responsible for the transport of the ferrous iron across the apical membrane of the enterocytes, as indicated in Figure 1.1 (Fleming et al. 1997, Gunshin et al. 1997). It has been observed that the amount of DMT1 and CYBRD1 found within the enterocytes increase significantly in conditions of iron deficiency (Gunshin et al. 1997, McKie et al. 2001)

Inside the enterocytes, iron is enzymatically liberated from haem by haem oxygenase. The inorganic iron may then follow one of two paths: (1) it may either be stored inside ferritin or (2) it may be transferred across the basolateral membrane surface to the plasma ferritin. The solute carrier family 40 (iron-regulated transporter) member 1 protein [SLC40A1, also known as the solute carrier family 11 (proton-coupled divalent metal ion transporter) member 3 protein (SLC11A3), ferroportin 1 (FPN1), iron-regulated transporter 1 (IREG1) or metal transporter 1 (MTP1)] is the major molecule mediating the transport of iron across the basolateral membrane (Abboud and Haile 2000, Donovan et al. 2000, McKie et al. 2000). SLC40A1 is induced by iron deficiency and localises to the basolateral membrane of polarised cells. Hephaestin (HEPH), a multicopper membrane ferroxidase, aids SLC40A1 in basolateral iron transport (Vulpe et al. 1999). The precise role HEPH portrays in the transfer of iron is unknown, but it is possible that during the transmembrane transfer process it

oxidises iron and/or is involved in the process of iron loading onto the plasma transferrin (Donovan and Andrews 2004). HEPH is similar to ceruloplasmin (CP), a plasma ferroxidase that possibly facilitates both the export of iron from the enterocyte, as well as its binding to transferrin (Hellman and Gitlin 2002).

Figure 1.1. A schematic representation of the pathways of iron absorption by the enterocyte.



Legend to Figure 1.1.

Dietary iron in the gut lumen is reduced from the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) state by CYBRD1. Fe^{2+} is transported into the enterocyte and is degraded by HMOX1 to release inorganic iron. The intracellular iron has two possible fates: 1) it may be stored as ferritin or 2) it may be transported across the basolateral membrane and into the blood plasma by SLC40A1. HEPH facilitates the export of iron by SLC40A1. Abbreviations: CYBRD1, cytochrome b reductase; DMT1, divalent metal transporter; Fe^{3+}, ferric iron; Fe^{2+}, ferrous iron; HEPH, hephaestin; HMOX1, haem oxygenase; SLC40A1, solute carrier family 40 (iron-regulated transporter) member 1; TBI, transferrin-bound iron; TFR1, transferrin receptor 1; TFR2, transferrin receptor 2. Adapted from Trinder et al. 2002a.

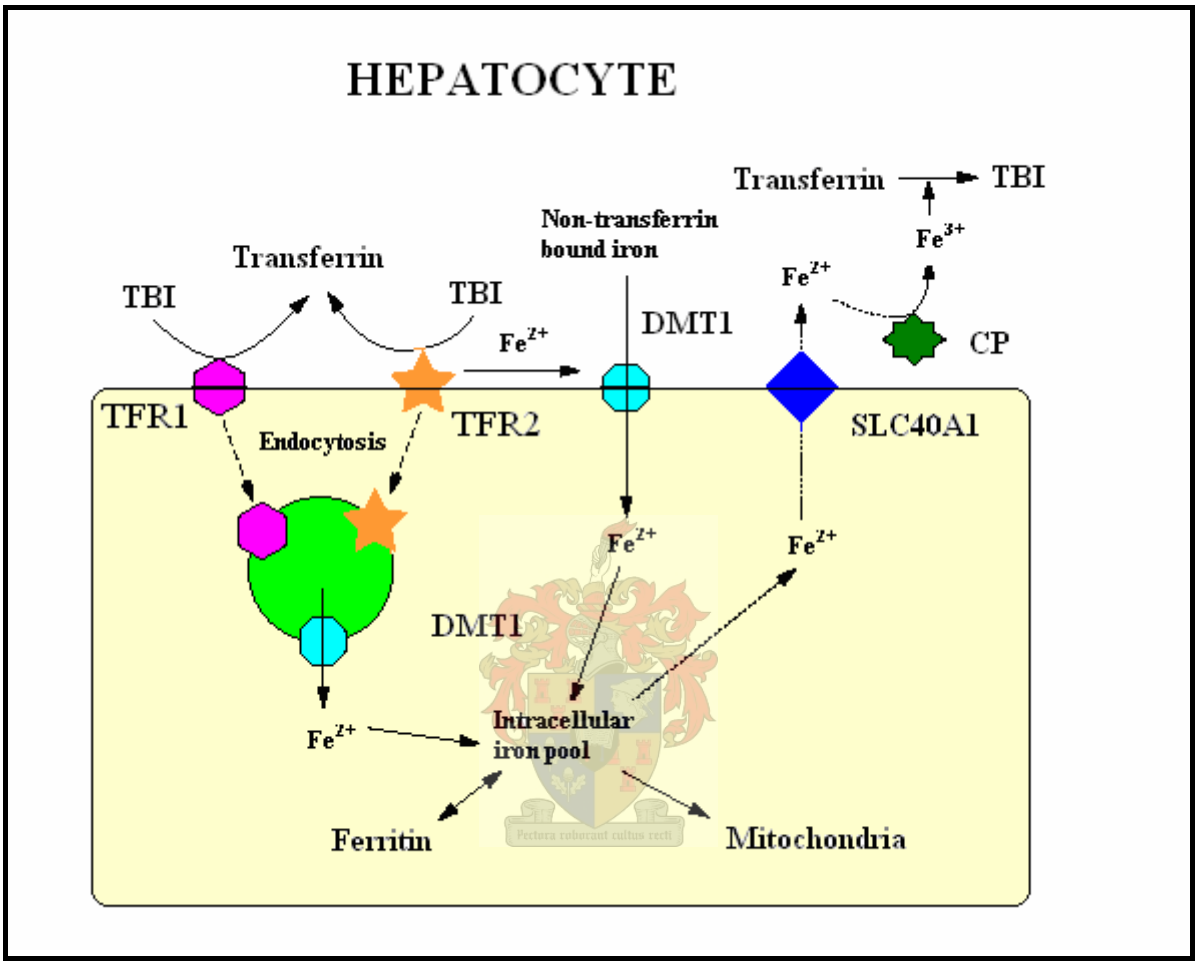
1.3.3. Macrophage iron recycling

Recovering iron from senescent red cells is a vital process as most of the body's iron is contained within the red blood cells. Damaged and old erythrocytes are phagocyted by macrophages, removing them from circulation. Erythrocyte lysis, as well as haemoglobin degradation occurs within the macrophages. Iron is then enzymatically released from haem by haem oxygenase. It is believed that SLC40A1 is responsible for the release of iron from the macrophages after erythrophagocytosis (Knutson et al. 2003, 2005). Ceruloplasmin is responsible for the oxidation of the released iron to the ferric state. Ferric iron can then be found bound to the circulating transferrin. It has been observed in patients suffering from anaemia of chronic disease (ACD) that the recycling of iron by the reticuloendothelial macrophages is defective, and intestinal iron absorption is also impaired.

1.3.4. Liver iron transport

Two transferrin receptors TFR1 and TFR2, mediate the uptake of transferrin-bound iron (TBI) by the liver (shown in Figure 1.2). HFE is expressed by hepatocytes and possibly regulates the uptake of TBI by TFR1. In iron-overloaded conditions, TFR1 expression is downregulated in hepatocytes and TFR2 expression, which is regulated by the transferrin saturation, is upregulated. Under these conditions, TFR2 may contribute to an increased uptake of TBI by the liver (Robb and Wessling-Resnick 2004). TFR2 has a higher capacity than TFR1 for transporting TBI into the liver. Iron transport from the hepatocytes is mediated by SLC40A1 (Abboud and Haile 2000).

Figure 1.2. A schematic representation of the pathways of iron uptake and the uptake of non-transferrin bound iron by the hepatocytes.



Legend to Figure 1.2.

Two transferrin receptors, TFR1 and TFR2, mediate the uptake of transferrin-bound iron (TBI) by the enterocyte. Iron transport from the hepatocytes, is mediated by SLC40A1. Abbreviations: CP, Ceruloplasmin; *DMT1*, divalent metal transporter 1; Fe^{3+}, ferric iron; Fe^{2+}, ferrous iron; SLC40A1, solute carrier family 40 (iron-regulated transporter) member 1; TBI, transferrin-bound iron; TFR1, transferrin receptor 1; TFR2, transferrin receptor 2. Adapted from Trinder et al. 2002a.

1.3.5. Iron storage

1.3.5.1. Hepatic iron storage

The liver is the major site of iron storage. The majority of stored iron is deposited in hepatocytes as either ferritin or haemosiderin (Trinder et al. 2002a). The transferrin-bound iron circulates through the liver portal system and is transferred to the hepatocytes via the transferrin receptor (Kawabata et al. 1999).

1.3.5.2. Reticoendothelial (RE) iron storage

Two mechanisms exist whereby the RE macrophages acquire iron. Firstly, iron is acquired via the surface transferrin receptors (Testa et al. 1991) and secondly, the macrophages acquire iron through the process of erythrophagocytosis (Deiss 1983). As mentioned previously, iron is enzymatically liberated from the enterocyte haem by haem oxygenase within the cells and at this point it is either released into the plasma with the aid of SLC40A1 or the iron is retained and stored as ferritin.

Ferritin, a nanobox protein, exists in two subunits known as the heavy and light chains. These chains form a protein shell with the ability of binding approximately 4000-4500 iron atoms (Aisen et al. 2001, Arosio and Levi 2002). Ferritin proteins contain catalytic sites for the oxidation of iron and hydrophilic pores enabling the exchange with the solvent. Iron is kept separated from the nucleus and other organelles. A fraction of ferritin can be found in serum and secretory fluids in vertebrates (Arosio and Levi 2002).

1.3.6. Regulation of iron homeostasis

Iron absorption is dependent on three factors: the level of body iron stores, the rate of erythropoiesis and hypoxia (Trinder et al. 2002a). Two models exist that explain the regulation of iron absorption: 1) the crypt-programming model and 2) the hepcidin model (Pietrangelo 2004).

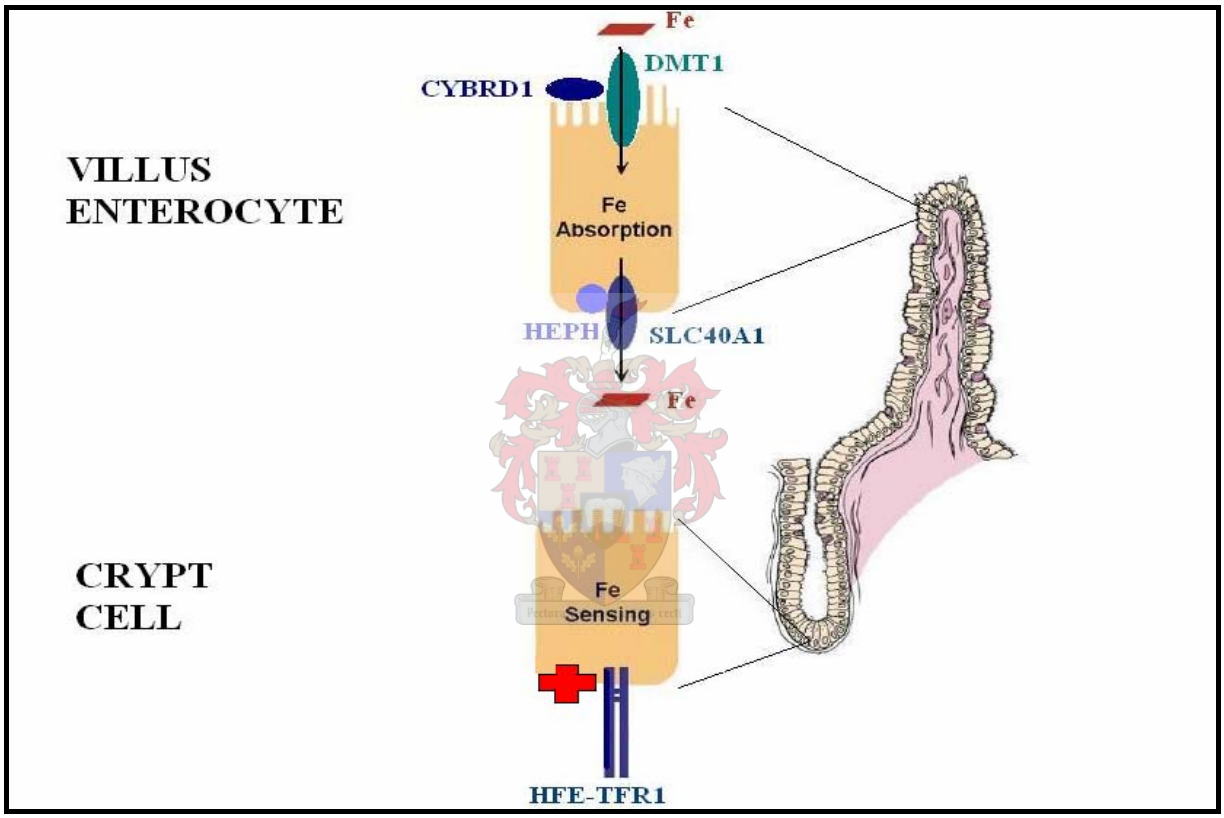
The crypt-programming model

This model proposes that the body iron levels can be sensed by the crypt cells, thus regulating the absorption of dietary iron by mature villus enterocytes (illustrated in Figure 1.3). The enterocytes found in the crypts of the duodenum are responsible for iron uptake from the plasma. The level of body iron stores matches the intracellular iron levels of the crypt cells and this subsequently determines the amounts of iron that is absorbed from the gut lumen as these crypt cells migrate toward the brush border (Oates et al. 2000). Both TFR1 and TFR2 proteins, which mediate the cellular uptake of TBI from the plasma, are expressed in the crypt cells.

It has been demonstrated that *HFE* is highly expressed in the crypt cells (Parkkila et al. 1997) and that the HFE protein is physically associated with TFR inside crypt enterocytes (Waheed et al. 1999). Waheed et al. (1999) hypothesized that by modulating the transferrin-mediated uptake of plasma iron, the wild-type HFE protein subsequently influences the ability of the crypt cells to sense the iron status of the body. The HFE protein is thus involved in the regulation of the mechanism that determines the amount of dietary iron the crypt enterocytes will absorb when they mature into villus enterocytes. The *Hfe* knockout mouse model of

Trinder et al. 2002 demonstrated that the mutant *HFE* is unable to facilitate the TFR1-mediated uptake of plasma iron. This observation adds support to the hypothesized crypt cell model, in which *HFE* regulates the uptake of TBI from the plasma by the duodenum.

Figure 1.3. A schematic representation of the duodenal crypt-programming model.



Legend to Figure 1.3

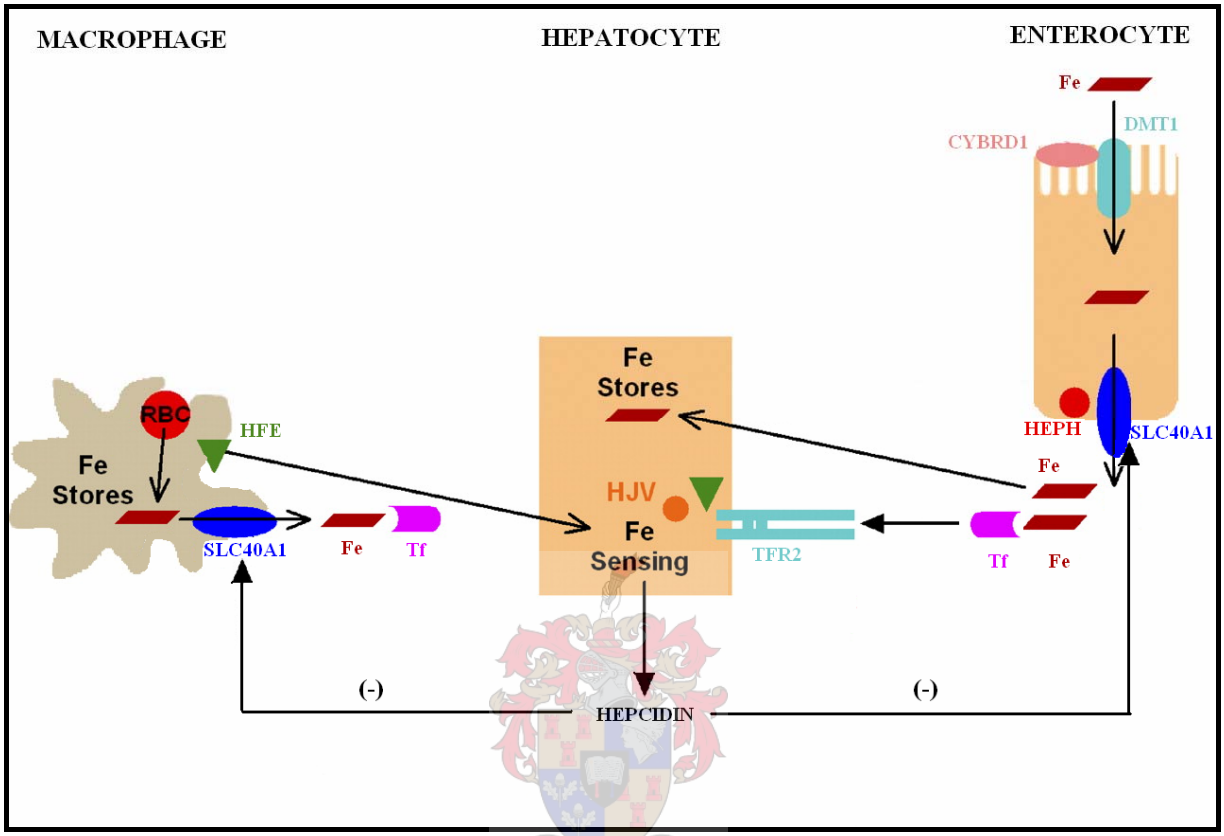
Duodenal villus cells are the major sites of iron absorption from the diet. Ferric iron is reduced to ferrous iron by theCYBRD1 protein on the luminal surface of villus cells. Villus enterocytes differentiate from crypt cells during migration from the crypts to the apex of the villus. The crypt cells may sense plasma iron *via* the HFE- TFR1 complex on the basolateral surface and program the level of expression of the iron transport genes expressed on differentiation of these cells to villus absorptive enterocytes. Abbreviations: CYBRD1, cytochrome b reductase; DMT1, divalent metal transporter; Fe, iron; HEPH, hephaestin; HFE, high iron protein; SLC40A1, solute carrier family 40 (iron-regulated transporter) member 1; TFR1, transferrin receptor 1. Adapted from Fleming and Sly 2002.

The Hepcidin model

Hepcidin is an antimicrobial peptide almost exclusively synthesised by the hepatocytes, thus being predominantly expressed in the liver. Not only does hepcidin perform the functions of a stores regulator, but it can also act as an erythroid regulator (Ganz 2003). When hepcidin levels are low, increased absorption of iron is triggered from the duodenum, as well as the release of iron from the macrophages (illustrated in Figure 1.4). When an excess of hepcidin is detected, iron absorption is decreased and iron is retained within the macrophages. The hepcidin levels are thus a reflection of the body iron stores (Papanikolaou and Pantopoulos 2005). The loss of the HFE protein causes a decrease in hepcidin levels, leading to a subsequent increase of ferroportin-mediated iron efflux from RE cells and duodenal enterocytes.

Non-transferrin-bound iron (NTBI) enters into the circulation when transferrin becomes saturated due to increasing iron levels in the circulation. The NTBI is transported to tissues with a high affinity for NTBI. Increased hepcidin expression has been shown to contribute to the development of anaemia of chronic disease (Weiss 2002), a condition characterised by hypoferremia, which is caused by the retention of iron within the macrophages. The expression levels of hepcidin were increased in mice and humans with inflammation (Nicolas et al. 2002, Nemeth et al. 2004) and suppressed in HH (Bridle et al. 2003).

Figure 1.4. A schematic representation of the hepcidin model.



Legend to Figure 1.4.

The interaction between transferrin (Tf) and the hepatocyte transferrin receptor, transduces a signal that leads to an increase in the expression of hepcidin in the hepatocyte. Hepcidin acts on target cells when secreted into the blood. With the decrease in iron export, there is an increase in iron stores and there is a subsequent decrease in dietary iron absorption in the duodenal enterocytes. Iron homeostasis is maintained when the circulating levels of Tf are normalised. Low hepcidin levels leads to increased iron absorption and also leads to iron release from the macrophages. Excessive hepcidin levels leads to decreased iron absorption. Abbreviations: CYBRD1, cytochrome b reductase; DMT1, divalent metal transporter; Fe, iron; HEPH, hephaestin; HFE, high iron protein; HJV, hemojuvelin protein; RBC, red blood cell; SLC40A1, solute carrier family 40 (iron-regulated transporter) member 1; Tf, transferrin; TFR2, transferrin receptor 2. Adapted from Fleming and Britton 2006.

1.4. Genes involved in iron homeostasis

Various genes have been implicated in iron transport and storage. These genes include the solute carrier family 11 (proton-coupled divalent metal ion transporter) member 2 gene [*SLC11A2*, also known as the natural resistance-associated macrophage protein 2 (*NRAMP2*) gene or divalent metal transporter 1 (*DMT1*) gene] (Gruenheid et al. 1997, Gunshin et al. 1997); the high iron (*HFE*) gene (Simon et al. 1976, Feder et al. 1996); haem oxygenase 1 (*HMOX1*) gene (Tenhunen et al. 1969), the hephaestin (*HEPH*) gene (Vulpe et al. 1999, Kaplan and Kushner 2000, Anderson et al. 2002, Petrak and Vyoral 2005); the solute carrier family 40 (iron-regulated transporter) member 1 gene [*SLC40A1*, also known as the solute carrier family 11 (proton-coupled divalent metal ion transporter) member 3 (*SLC11A3*) gene, ferroportin 1 (*FPN1*) gene, iron-regulated transporter 1 (*IREG1*) gene or metal transporter 1 (*MTP1*) gene (Abboud and Haile 2000, Donovan et al. 2000, McKie et al. 2000); the hepcidin antimicrobial peptide gene [*HAMP*, also known as the liver-expressed antimicrobial peptide 1 (*LEAP1*) gene or the hepcidin (*HEPC*) gene] (Krause et al. 2000, Nicolas et al. 2001, Park et al. 2001, Pigeon et al. 2001); the transferrin receptor 2 (*TFR2*) gene (Camaschella et al. 2000, Roetto et al. 2001); the cytochrome b reductase 1 gene [*CYBRD1*, also known as the duodenal cytochrome b (*DCYTB*) gene] (McKie et al. 2001); the ceruloplasmin (*CP*) gene (Cairo et al. 2001) and the hemojuvelin (*HJV*) gene (Papanikolaou et al. 2004).

Genes investigated in this study include *HFE*, *HMOX1*, *SLC40A1*, *HAMP, CYBRD1* and *HJV* and only these genes will be discussed further.

1.4.1. High iron (*HFE*) gene

The gene associated with haemochromatosis was first mapped to chromosome 6 (Simon et al. 1976), but it was not until 1996 that the *HFE* gene was localised to chromosome 6 (6p21.3) and isolated using positional cloning (Feder et al. 1996). The gene consists of seven exons and encodes a 343 amino acid protein that comprises three extracellular domains (the α1, α2 and α3 loops), a transmembrane domain and a cytoplasmic tail. *HFE* forms a heterodimer with β_2-microglobulin for cell surface expression. The primary structure of *HFE* is homologous to HLA-A2, a MHC class I protein, and the non-classical class I protein HLA-G1 (Feder et al. 1996). However, it has been suggested that HFE does not have a functional peptide-binding groove (Lebron et al. 1998).

HFE and the transferrin receptor 1 (TFR1), form a high-affinity protein-protein complex. This complex reduces the affinity of TFR1 for transferrin approximately ten-fold, thereby reducing ferritin concentrations accordingly in mammalian cells (Parkkila et al. 1997, Feder et al. 1998, Gross et al. 1998, Bennett et al. 2000).

Targeted disruption of the mouse *Hfe* gene was studied in order to determine the involvement of this gene in the regulation of iron homeostasis. Profound differences in the parameters of iron homeostasis in Hfe-deficient mice were detected, leading to the conclusion that the Hfe protein is indeed involved in the regulation of iron homeostasis and that mutations in *HFE* cause hereditary haemochromatosis (HH) (Zhou et al. 1998).

HH is predominantly caused by the C282Y and H63D mutations, which represent two inherited base pair changes within the *HFE* gene (Feder et al. 1996). The C282Y missense

mutation produces a guanidine-to-adenine (G→A) transition at nucleotide position 845 of the *HFE* gene, causing a cysteine to tyrosine substitution at amino acid 282. This mutation is located in the α3-domain. Association of the HFE protein with β_2-microglobulin is disrupted, causing the subsequent misfolding of the protein (Feder et al. 1996, 1997). Homozygosity for this mutation is the most common cause of HH and in individuals of European descent it is responsible for 90% of cases (The UK Haemochromatosis Consortium 1997).

The H63D missense mutation results in a cytosine-to-guanine (C→G) transversion at nucleotide 187, causing a histidine to aspartic acid substitution at amino acid position 63 (Feder et al. 1996, 1997). HH patients heterozygous for C282Y are frequently heterozygous for H63D (Feder et al. 1996, 1997). This mutation accounts for 4.5% of HH cases (Merryweather-Clarke et al. 2000). Three other mutations found in *HFE* (S65C, I105T, G93R) are also associated with the development of iron overload (Barton et al. 1999).

1.4.2. Haem oxygenase 1 (*HMOX1)* gene

A human haem oxygenase (*HMOX1*) cDNA was isolated by screening a cDNA library with a rat cDNA. The cDNA library had been constructed using poly (A)-rich RNA from macrophages treated with hemin to increase haem oxygenase activity and mRNA levels (Yoshida et al. 1988). *HMOX1* was assigned to chromosome region 22q12 using fluorescent *in situ* hybridisation (FISH) analysis (Kutty et al. 1994). Seroussi et al. (1999) later characterised a contig containing five genes, including *HMOX1*, in human 22q13.1 and also mapped the mouse *Hmox1* gene to chromosome 8, using FISH analysis. Haem oxygenase, an essential enzyme in haem catabolism, cleaves haem to form biliverdin (Tenhunen et al. 1969), which is subsequently converted to bilirubin by biliverdin reductase (Tenhunen et al. 1970).

HMOX1, consisting of five exons, encodes a 288 amino acid protein. Similar to rat Hmox1, a putative membrane segment mainly composed of hydrophobic amino acids is found at the carboxyl terminus of human HMOX1. The human haem oxygenase, one amino acid shorter than rat Hmox, is 80% homologous to the rat amino acid sequence (Yoshida et al. 1988). HMOX1 contains no cysteine residues and contains six histidine residues of which five are conserved in rat Hmox1 (Shibahara et al. 1985, Yoshida et al. 1988).

In their studies of Wistar rats, Wagener et al. (2003) investigated haem and haem oxygenase involvement in the inflammatory process during wound healing. Haem accumulation was observed directly at the wound edges in the rat palate. This coincided with the recruitment of leukocytes, increased adhesion molecule expression and increased *Hmox1* expression upon inflammation. These results indicated that the release of haem possibly acts as a physiological trigger of inflammatory processes, while Hmox1 antagonises inflammation by reducing adhesive interactions and cellular infiltration (Wagener et al. 2003).

An analysis of the parameters of iron metabolism was done on mice with targeted *Hmox1* null mutations. The adult *Hmox1* deficient mice developed both serum iron deficiency and pathological iron loading, signifying Hmox1 is crucial for the expulsion of iron from tissue stores (Poss and Tonegwa 1997).

Various mutations and a promoter repeat of *HMOX1* have been implicated in various diseases, including neurodegenerative (Kimpara et al. 1997) and pulmonary diseases (Yamada et al. 2000). In addition to its role in haem degradation, *HMOX1* also plays a crucial role in the maintenance of cellular homeostasis (Maines 2000). There is also accumulating evidence

indicating that an excess of free haem could lead to cell damage and tissue injuries, since haem can catalyse the formation of ROS (Jeney et al. 2002).

1.4.3. Solute carrier family 40 (iron-regulated transporter), member 1 (*SLC40A1*) gene

Three independent groups are responsible for the discovery of *SLC40A1*. Using a positional cloning strategy, Donovan et al. (2000) identified the gene responsible for the severe anaemic phenotype, weissherbst, in zebrafish. The resulting ferroportin-1 cDNA of mice and humans were isolated by RT-PCR analysis of the liver and placenta, respectively (Donovan et al. 2000). McKie et al. (2000) used a subtractive cloning strategy, as well as PCR analysis, for the isolation of ferroportin cDNA from hypotransferrinaemic mice that absorb iron at very high rates. Abboud and Haile (2000) employed an iron-responsive protein (IRP) affinity column to fish out mRNAs containing iron-responsive elements (IREs). This method led to the identification of the metal transporter protein-1 (MTP1).

Fluorescent in situ hybridisation (FISH) was used to map the human *SLC40A1* gene to chromosome 2 (2q32) and the mouse homologue to chromosome 1B (Haile 2000). *SLC40A1* consists of eight exons and encodes a 571 amino acid protein containing ten transmembrane domains. The SLC40A1 protein is localised to the basolateral membrane of polarised epithelial cells (McKie et al. 2000). The 5' untranslated region of the mRNA contains a functional iron responsive element (IRE) predicted to form a hairpin-loop (McKie et al. 2000).

McKie et al. (2000) observed that the expression of *SLC40A1* stimulated the iron efflux in Xenopus oocytes; leading to the conclusion that SLC40A1 is the duodenal iron export protein upregulated in HH. SLC40A1 is expressed in various cell types, including the placental syncytiotrophoblasts, duodenal enterocytes, hepatocytes and the reticuloendothelial macrophages, all of which play critical roles in iron metabolism (Donovan et al. 2000).

Patients with mutations in *SLC40A1* have larger reticoendothelial iron stores than patients with other forms of haemochromatosis. It was proposed that the partial loss of *SLC40A1* function leads to abnormal iron homeostasis and consequently iron overload (Montosi et al. 2001). Reticuloendothelial iron overload in patients with ferroportin disease suggested that loss of function mutations in *SLC40A1* gene impair reticuloendothelial iron release (Cazzola et al. 2002).

Donovan et al. (2005) observed embryonic lethality in *Slc40a1* knockout mice. A mild disruption of iron homeostasis was detected in heterozygous mice. Although these mutant mice appeared normal at birth, they later developed anaemia and abnormal iron accumulation in duodenal enterocytes, Kupffer cells, hepatocytes and splenic macrophages. Slc40a1 was deleted in all tissues of the mutant mice, except from the extraembryonic visceral endoderm and the placenta. Severe iron deficiency anaemia developed in mice in which the Slc40a1 deletion was restricted to the intestines. These findings indicate the importance of Slc40a1 in prenatal and post-natal iron homeostasis (Donovan et al. 2005).

Various mutations in *SLC40A1* were reported and considerable clinical differences were observed between the reported families (Cazzola et al. 2002, Devalia et al. 2002, Roetto et al. 2002, Wallace et al. 2002, 2004, 2005, Arden et al. 2003, Jouanolle et al. 2003, Pietrangelo

2004, Cemonesi et al. 2005, Kohgo 2005, Morris et al. 2005, Sham et al. 2005). Both parenchymal and reticuloendothelial iron overload were detected in certain families suffering from ferroportin disease, while others only displayed a typical genetic reticuloendothelial iron overload (Cazzola 2003, Pietrangelo 2004). It appears that the different mutations detected in *SLC40A1* may be responsible for phenotypic variability among patients.

1.4.4. Hepcidin antimicrobial peptide (*HAMP)* gene

HAMP is a liver-expressed antimicrobial peptide (also termed *LEAP-1*) exhibiting antimicrobial activity. The cDNA encoding *HAMP* was isolated *via* biochemical purification of blood ultrafiltrate using a cysteine alkylation assay and mass spectometry, micropeptide sequencing, RT-PCR analysis and 5' and 3' rapid amplification of cDNA ends (RACE) (Krause et al. 2000).

The cDNA encoding *HAMP* was isolated by means of biochemical purification of hepcidin from urine, amino acid sequence analysis, EST database searching and 5' RACE (Park et al. 2001). The human *HAMP* gene was mapped to chromosome 19 (19q13) (Krause et al. 2000, Park et al. 2001, Pigeon et al. 2001), while the mouse gene was mapped to chromosome 7 (Pigeon et al. 2001).

HAMP consists of three exons (Krause et al. 2000, Park et al. 2001, Pigeon et al. 2001). This gene encodes an 84 amino acid propeptide with a 24-residue N-terminal signal sequence, as well as a penta-arginyl proteolysis site that is followed by the active C-terminal 25-amino acid peptide (Krause et al. 2000). The 84 amino acid protein can undergo enzymatic cleavage into mature 20, 22 and 25 amino acids. Exon 3 of the gene encodes the active peptide, which

contains a unique 17-residue stretch with eight cysteines forming the intramolecular disulfide bridges lending stability to the beta-sheet structure (Krause et al. 2000, Pigeon et al. 2001).

Experiments were performed on a mouse model exhibiting iron overload caused by targeted disruption of the upstream stimulatory factor 2 (*Usf2*) gene. These experiments detected the inhibition of *Hamp* expression in these mice (Nicolas et al. 2001). Furthermore, the development of iron overload in these mice exhibited a striking resemblance to that observed in human HH patients and in *HFE* knockout mice (Zhou et al. 1998, Bahram et al. 1999, Levy et al. 1999).

HAMP has the ability to regulate the SLC40A1 protein levels, by inducing its internalisation and lysosomal degradation (Nemeth et al. 2004). Mutations identified in *HAMP* have also been associated with juvenile haemochromatosis (Roetto et al. 2003).

1.4.5. Cytochrome b reductase 1 (*CYBRD1*) gene

A gene encoding a cytochrome b-like molecule was isolated from hypotransferrinaemic mice by subtractive cloning. *CYBRD1* is comprised of four exons and encodes a 286 amino acid protein with six transmembrane domains and four conserved histidine residues (McKie et al. 2001). The *CYBRD1* gene was mapped to chromosome 2 by the International Radiation Hybrid Mapping Consortium.

CYBRD1 shares 40-50% homology with cytochrome b561, a b-type haem plasma ferric reductase that is involved with the regeneration of ascorbic acid from dehydroascorbate. The gene catalyses the reduction of ferric (Fe^{3+}) to ferrous iron (Fe^{2+}) in the gut lumen during

intestinal iron absorption and is highly expressed at the intestinal brush border. Although *CYBRD1* contains no iron responsive elements, its expression is very strongly regulated by iron (McKie et al. 2001).

CYBRD1 mRNA and protein levels are elevated in conditions such as hypoxia and iron deficiency, which stimulate intestinal iron absorption, and reduced in conditions of iron loading (McKie et al. 2001, 2002). Gunshin et al. (2005) suggested that *CYBRD1* is not required for intestinal iron absorption in mice that received a normal iron diet.

1.4.6. Hemojuvelin *(HJV)* gene

Using a positional cloning approach, the hemojuvelin (*HJV*) gene, associated with juvenile haemochromatosis (JH), was identified within chromosome 1 (1q21). *HJV* consists of four exons, has a length of 4 265 bp and encodes a 426 amino acid protein. The gene is transcribed into a full-length transcript with five alternatively spliced transcripts. The HJV protein displays homology to a repulsive guidance molecule (RGM) and also contains several functional sequence motifs that share homology with other proteins. These include a signal peptide, a RGD (arginine-glycine-aspartic acid) motif, a von Willebrand factor type D domain and a putative glycosyl-phosphatidylinositol (GPI) anchor site (Papanikolaou et al. 2004).

Northern Blot analysis of human tissue revealed that similar to *HAMP*, expression of the *HJV* transcript was restricted to the liver, skeletal muscle and the heart (Papanikolaou et al. 2004). A recent study detected *HJV* expression in parts of the colon, the pancreas and the oesophagus (Rodriguez Martinez et al. 2004). The reduced urinary hepcidin levels in patients with 1q-linked JH is an indication that hemojuvelin probably acts as a modulator of hepcidin

expression rather than a hepcidin receptor (Lanzara et al. 2004, Papanikolaou and Pantopoulos 2004). The functional relationship of *HJV* with *HAMP*, as well as the role of *HJV* in iron regulation remains uncertain.

Niederkofler et al. (2005) detected *Hjv* expression by the periportal hepatocytes in the mouse liver. The *Hjv* mutant mice displayed conditions of iron overload and no expression of hepcidin, in response to dietary iron or the injected iron, was detected. The ability to upregulate hepcidin in response to acute inflammation was conserved, when induced by the lipopolysaccharide or its downstream products. *Hjv* expression was downregulated in the liver, but not in skeletal muscle after inflammation was induced in wild-type mice (Niederkofler et al. 2005).

Huang et al. (2005) observed that there was a rapid accumulation of iron in the liver, pancreas and heart of *Hjv* mutant mice, but the iron levels were decreased in the spleen. Unlike human patients, no abnormalities in fertility were detected and there were also no apparent cardiac or endocrine abnormalities. This could suggest that these mice display more resistance to end-organ damage. In the intestinal epithelial cells as well as the macrophages, there was a marked decrease in the hepatic hepcidin expression and an increase in the SLC40A1 protein levels (Huang et al. 2005).

1.5. Objectives of this study

Identification of genetic risk factors that contribute to OC susceptibility could lead to a better understanding of the disease pathogenesis. Early diagnosis and calculation of risk can lead to improved counselling and treatment and thus be of great importance to OC individuals.

The aims of this study were as follows:

- The mutation analysis of six genes (including *HFE*, *HMOX1*, *SLC40A1*, *HAMP*, *CYBRD1* and *HJV*) involved in iron metabolism by:
    - PCR amplification of the coding and flanking intronic regions of these genes
    - Heteroduplex-single strand conformation polymorphism (HEX-SSCP) analysis, restriction fragment length polymorphism (RFLP) analysis and semi-automated DNA sequencing analysis of the amplified products for the identification of any novel and/or previously described variants in these genes.
- The variants detected were subjected to statistical analysis
    - to identify the possible existence of significant associations that could link the variants to OC susceptibility and
    - to establish possible gene-gene interaction.

Chapter 2

Detailed Experimental Procedures

The study protocol was approved by the Ethics Review Committee of the University of Stellenbosch (Project number 2002/C071).

2.1 Subjects

The study population included only individuals from the Black Xhosa-speaking population of South Africa, referring to South Africans of central African descent with cultural habits originating from Xhosa tribes.

Blood samples were obtained with written informed consent from 50 (25 male and 25 female) unrelated patients with squamous cell carcinoma of the oesophagus. Patients with OC display symptoms such as excessive weight loss and difficulty to swallow. These patients were referred to the Provincial Hospital, Port Elizabeth, where they were subjected to a barium swallow followed by biopsies in the theatre to confirm the presence of an ADC or SCC on the basis of the histology (Hermanek and Sobin 1987).

Blood samples were obtained with written informed consent from 50 healthy, unrelated population-matched individuals as controls. Control individuals were recruited at the Mbekweni and Mpeko Clinics, Transkei. The control individuals were subjected to early screening for OC by brush biopsy, using a Nabeya capsule.

2.2 Body iron status

Iron overload was defined as the percentage transferrin saturation (% TS) > 45 and/or serum ferritin > 200 μg/l for females and > 300 μg/l for males (Looker and Johnson 1988, Adams and Chakrabarti 1998). Four categories of iron status were established: iron deficiency (serum ferritin < 20 μg/l), normal ferritin levels (females: 20–200 μg/l and males: 20-300 μg/l), raised serum ferritin (females: > 200 μg/l and males: > 300 μg/l) with %TS < 45 and raised serum ferritin with %TS > 45.

2.3 Total genomic isolation from whole blood

Total genomic DNA was extracted from whole blood samples using a modified protocol of Miller et al. (1988).

Whole blood was collected in ethylenediamine tetra-acetic acid (EDTA) tubes for preservation, and stored at -20ºC prior to DNA extraction. The thawed blood was decanted into a 50 ml polypropylene Falcon tube to which 40 ml cold lysis buffer [155 mM ammonium chloride (NH_4Cl), 10 mM potassium hydrogen carbonate ($KHCO_3$), 0.1 mM EDTA, pH 7.4] was added. The solution was incubated on ice for 30 minutes until the red blood cells had undergone lysis, followed by centrifugation at 250 x *g* for 30 minutes. Subsequent to centrifugation, the supernatant was discarded and the pellet washed in 20 ml phosphate buffered saline solution (PBS). The solution was centrifuged at 250 x *g* for 20 minutes. The supernatant was discarded and the pellet resuspended in 3 ml nuclear lysis buffer [10 mM Tris hydrochloride (Tris-HCl), 400 mM sodium chloride (NaCl), 2 mM EDTA, pH 8.2], 1.5

mg/ml proteinase K (Roche Diagnostics), and 1% (w/v) sodium dodecyl sulphate (SDS) and incubated overnight in a 55ºC water bath.

Following overnight incubation, 6 M NaCl was added to the solution. The solution was shaken vigorously for one minute, followed by centrifugation at 950 x *g* for 15 minutes. The supernatant was transferred to a fresh 50 ml Falcon tube and the remaining pellet and foam were discarded. Centrifugation was performed at 950 x *g* for 15 minutes and the supernatant subsequently transferred to a clean Falcon tube. Two volumes of ice-cold ± 99.9% (v/v) ethanol (EtOH) were added to the supernatant. The spool of DNA formed was transferred to a fresh 1.5 ml Eppendorf tube and centrifuged at 950 x *g* in 1 ml 70% (v/v) EtOH for 15 minutes for the removal of excess salt. After the EtOH was discarded, the pellet was allowed to air dry and resuspended in 500 µl sterile SABAX water (Adcock Ingram, South Africa). The suspension was shaken overnight at room temperature to allow the DNA to dissolve completely. The DNA concentrations were determined using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies), according to the manufacturer's protocol. The DNA was subsequently diluted to a concentration of 50 ng/µl and stored at 4ºC.

2.4 Polymerase chain reaction (PCR) amplification

2.4.1 Oligonucleotide primers

The PCR primers used in this study are listed in Tables 2.1 - 2.6. Primers were designed using the Primer3 program (Rozen and Skaletsky 2000, available http://www-genome.wi.mit.edu/cgi-bin/primer/primer3/www.cgi,2002) and manufactured by Inqaba Biotech (Pretoria, South Africa). The melting temperature (T_M) for each of the primers was

calculated according to the equation described by Thein and Wallace (1986). The equation is as follows: $T_M = 2(nA + nT) + 4(nG + nC)$. PCR conditions were optimised for each primer set and conditions are indicated in Tables 2.1 - 2.6.

Table 2.1. Oligonucleotide primers used for PCR amplification of the *HFE* gene

Exon	Primer name	Primer sequences 5' - 3'	T_M (ºC)	Amplicon size (bp)	T_A (ºC)	PCR cycle	$MgCl_2$ (mM)
1	HFE-1F[a]	TTACTGGGCATCTCCTGAGC	62	256	55	C	1.5
	HFE-1R[a]	CTAGTTTCGATTTTTCCACCCC	61				
2	HFE-2FA[b]	ACATGGTTAAGGCCTGTTGC	60	298	55	C	1.5
	HFE-2RA[a]	TACCCTTGCTGTGGTTGTGA	60				
	HFE-2FB[a]	TGACCAGCTGTTCGTGTTCT	60	257	55	C	1.5
	HFE-2RB[b]	CAGCTGTTTCCTTCAAGATGCA	61				
3	HFE-3FA[a]	CTTGGGGATGGTGGAAATAG	60	279	57	C	1.5
	HFE-3RA[a]	CTCCAGGTAGGCCCTGTTCT	65				
	HFE-3FB[a]	CGAGGGCTACTGGAAGTACG	65	280	57	C	1.5
	HFE-3RB[a]	CTGCAACCTCCTCCACTCTG	65				
4	HFE-4F[b]	TGGCAAGGGTAAACAGATCC	60	390	57	C	1.5
	HFE-4R[b]	CTCAGGCACTCCTCTCAACC	65				
5	HFE-5F[a]	GAGAGCCAGGAGCTGAGAAA	62	297	55	C	1.5
	HFE-5R[b]	CAGAGGTACTAAGAGACTTC	58				
6	HFE-6F[b]	TAGTGCCCAGGTCTAAATTG	58	202	57	C	1.5
	HFE-6R[b]	TGAGTCTCTAGTTTTGTCTCC	59				

Abbreviations: 5', 5-prime; 3', 3-prime; ºC, degrees Celsius; bp, base pair; F, forward primer; *HFE* , high iron gene; mM, millimoles per liter; $MgCl_2$, magnesium chloride; PCR, polymerase chain reaction; R, reverse primer; T_A, annealing temperature; T_M, melting temperature. $T_M = 2(nA+nT)+4(nG+nC)$. References: [a] This study, [b] Prof C Camaschella.

Table 2.2. Oligonucleotide primers used for PCR amplification of the *HMOX1* gene

Exon	Primer name	Primer sequences 5' - 3'	T_M (ºC)	Amplicon size (bp)	T_A (ºC)	PCR cycle	$MgCl_2$ (mM)
1	HMOX1-1F[a]	CCGCCGAGCATAAATGTG	60	300	60	A	1.5
	HMOX1-1R[a]	GCACAGGCAGGATCAGAAC	62				
2	HMOX1-2F[b]	CAGCCAGCTTTGTGTTCACC	72	236	60	A	1.5
	HMOX1-2R[b]	AACCACTGGTCTGAGCCTTG	70				
3A	HMOX1-3FA[b]	TAGTGGACGGGACGGACAGA	74	187	60	A	1.5
	HMOX1-3RA[b]	CCTTGCGGTGCAGCTCTTCT	76				
3B	HMOX1-3FB[b]	TGAGCGCAACAAGGAGAGCC	76	314	60	A	1.5
	HMOX1-3RB[b]	GGAAGGTGAAGAAGGCCAGG	74				
3C	HMOX1-3FC[b]	GCCTGGCCTTCTTCACCTTC	74	276	60	A	1.5
	HMOX1-3RC[b]	TGGCAGTGCTGGAACTCTGG	75				
4	HMOX1-4F[b]	GGACCTGGTAGCATCTCTCA	67	314	60	A	1.5
	HMOX1-4R[b]	GCGAGAACCTGTCCTTACAG	68				
5A	HMOX1-5FA[b]	CCACCTGTTAATGACCTTGC	68	343	60	A	1.5
	HMOX1-5RA[b]	GAAGATGCCATAGGCTCCTT	69				
5B	HMOX1-5FB[b]	GGAAGGAGCCTATGGCATCT	71	303	60	A	1.5
	HMOX1-5RB[b]	GCTGAGCCAGGAACAGAGTG	74				
5C	HMOX1-5FC[b]	CACTCTGTTCCTGGCTCAGC	71	254	60	A	1.5
	HMOX1-5RC[b]	CTCCTACCGAGCACGCAAGA	74				

Abbreviations: 5', 5-prime; 3', 3-prime; ºC, degrees Celsius; bp, base pair; F, forward primer; *HMOX1* , haem oxygenase gene; $MgCl_2$, magnesium chloride; mM, millimoles per liter; PCR, polymerase chain reaction; R, reverse primer; T_A, annealing temperature; T_M, melting temperature. $T_M = 2(nA+nT)+4(nG+nC)$. References:[a] F Booley (unpublished data); [b]H Waso (unpublished data).

Table 2.3. Oligonucleotide primers used for PCR amplification of the *SLC40A1* gene

Exon	Primer name	Primer sequences 5' - 3'	T_M (ºC)	Amplicon size (bp)	T_A (ºC)	PCR cycle	$MgCl_2$ (mM)
1A	SLC40A1-1FA[a]	CCAGTCGGAGGTCGCAGG	67	318	60	A	1.5
	SLC40A1-1RA[a]	CAGGAGTGCAAGGAACTGG	62				
1B	SLC40A1-1FB[a]	CCAAAGTCGTCGTTGTAGTC	60	276	55	C	1.5
	SLC40A1-1RB[a]	TTCCTCCAGAACTCGTGTAG	60				
2	SLC40A1-2F[b]	TGGATAAGCATTCTGCCCTC	60	275	55	C	1.5
	SLC40A1-2R[b]	AAAGCATGTGTACTTGGATG	56				
3	SLC40A1-3F[a]	GATAAGGAAGCAACTTCCTG	58	339	55	C	1.5
	SLC40A1-3R[a]	CCTGGTTGTTTCTCTCCTAG	60				
4	SLC40A1-4F[b]	GGATAAGAACAGTCTCACTG	58	243	55	C	1.5
	SLC40A1-4R[b]	TTCATCCTTTACCACTACCAG	60				
5	SLC40A1-5F[b]	TTAAACTGCCTTGTTTAGTG	54	278	55	C	1.5
	SLC40A1-5R[b]	GCCTCATTTATCACCACCG	58				
6	SLC40A1-6F[a]	TTGTGTAAATGGGCAGTCTC	58	368	55	C	1.5
	SLC40A1-6R[a]	CATTTAAGGTCTGAACATGAG	57				
7A	SLC40A1-7FA[a]	GCTTTTATTTCTACATGTCC	54	352	60	A	1.5
	SLC40A1-7RA[a]	CCAGTTATAGCTGATGCTC	58				
7B	SLC40A1-7FB[a]	GGGTACGCCTACACTCAG	62	298	55	C	1.5
	SLC40A1-7RB[a]	CAGTTGTAATTTCAGGTATC	54				
7C	SLC40A1-7FC[a]	GAAGATATCCGATCAAGGTTC	59	259	55	C	1.5
	SLC40A1-7RC[a]	TTAATGGATTCTCTGAACCTAC	57				
8A	SLC40A1-8FA[a]	TTGAAATGTATGCCTGTAAAC	55	343	55	C	1.5
	SLC40A1-8RA[a]	TTCCTTCCTAACTTCTTTTGC	57				
8B	SLC40A1-8FB[a]	CCGATTTGCCCAAAATACTC	58	297	55	C	1.5
	SLC40A1-8RB[a]	TTTCCATGCCTCAACATAAGG	59				
8C	SLC40A1-8FC[a]	GTTTTTACCACAGCTGTGCC	60	359	55	C	1.5
	SLC40A1-8RC[a]	GTCTTCATACTTGAAGAATTTG	55				

Abbreviations: 5', 5-prime; 3', 3-prime; ºC, degrees Celsius; bp, base pair; F, forward primer; $MgCl_2$, magnesium chloride; mM; millimoles per liter; PCR, polymerase chain reaction; R, reverse primer; *SLC40A1* , solute carrier family 40 (iron-regulated) member 1 gene; T_A, annealing temperature; T_M, melting temperature. $T_M = 2(nA+nT)+4(nG+nC)$. References: [a]L Bloem (unpublished data), [b]Njajou et al. (2001).

Table 2.4. Oligonucleotide primers used for PCR amplification of the *HAMP* gene

Exon	Primer name	Primer sequences 5' - 3'	T_M (ºC)	Amplicon size (bp)	T_A (ºC)	PCR cycle	$MgCl_2$ (mM)
1	HAMP-1F[a]	AGCAAAGGGGAGGGGGCTCAGACC	71	262	60	A	1.5
	HAMP-1R[a]	TCCCATCCCTGCTGCCCTGCTAAG	70				
2	HAMP-2F[b]	AAACCACTTGGAGAGGAGCA	60	235	55	A	1.5
	HAMP-2R[b]	GAAGGAAGGGAATGTGAGCA	60				
3	HAMP-3F[b]	GCAACAGTGATGCCTTTCCT	60	272	55	A	1.5
	HAMP-3R[b]	CCAGCCATTTTATTCCAAGACC	61				

Abbreviations: 5', 5-prime; 3', 3-prime; ºC, degrees Celsius; bp, base pair; F, forward primer; *HAMP* , hepcidin antimicrobial peptide gene; $MgCl_2$, magnesium chloride; mM, millimoles per liter; PCR, polymerase chain reaction; R, reverse primer; T_A, annealing temperature; T_M, melting temperature. $T_M = 2(nA+nT)+4(nG+nC)$. References: [a]Dr A Merryweather-Clarke, [b]This study

Table 2.5. Oligonucleotide primers used for PCR amplification of the *CYBRD1* gene

Exon	Primer name	Primer sequences 5' - 3'	T_M (ºC)	Amplicon size (bp)	T_A (ºC)	PCR cycle	$MgCl_2$ (mM)
1	CYBRD1-1F[a]	GAGACAGCCCCAAGAAGTCG	65	378	61	B	2
	CYBRD1-1R[a]	TTCACGGAGGACCCTCTGCC	67				
2	CYBRD1-2F[a]	CCAGTGTGTCAAACTGTTC	58	346	51	B	2
	CYBRD1-2R[a]	CATTTACAGTCTGAATTG	54				
3	CYBRD1-3F[a]	TTGTCATACACATATTGC	53	318	50	B	2
	CYBRD1-3R[a]	CATTTTCCCAGTGAACAAGTA	57				
4A	CYBRD1-4FA[a]	GCATGTTGCTGTATCATCCTGT	61	254	57	B	2
	CYBRD1-4RA[a]	AGAGTAGGCTGGCATGGAAC	62				
4B	CYBRD1-4FB[a]	AAATGGAGGCACTGAACAGG	60	217	56	B	2
	CYBRD1-4RB[a]	AGGAGAAGCAAAACTGTAGAGC	61				

Abbreviations: 5', 5-prime; 3', 3-prime; ºC, degrees Celsius; bp, base pair; *CYBRD1* , cytochrome b reductase 1 gene; F, forward primer; $MgCl_2$, magnesium chloride; mM, millimoles per liter; PCR, polymerase chain reaction; R, reverse primer; T_A, annealing temperature; T_M ,melting temperature. $T_M = 2(nA+nT)+4(nG+nC)$. References:[a] F Booley (unpublished data)

Table 2.6. Oligonucleotide primers used for PCR amplification of the *HJV* gene

Exon	Primer name	Primer sequences 5' - 3'	T_M (ºC)	Amplicon size (bp)	T_A (ºC)	PCR cycle	$MgCl_2$ (mM)
1	HJV-1F	TCTGGCCAGCCATATACTCC	62	293	58	B	2
	HJV-1R	CAGCATTTGGACGAGACA	58				
2	HJV-2F	CACTCCACATTATCCTTACC	58	284	56	B	2
	HJV-2R	ATGCCCACCCCTACATAGC	62				
3A	HJV-3FA	ACACTCCGATAGAGCAGAGG	62	298	56	B	2
	HJV-3RA	TCTTCGATGCCATGTACCG	60				
3B	HJV-3FB	TAGAGGTGGGGGTTCATCAG	60	300	58	B	2
	HJV-3RB	CGGCCTTCATAGTCACAAGG	62				
3C	HJV-3FC	GACCTGATGATCCAGCACAA	62	287	56	B	2
	HJV-3RC	TGGCTTGGACAAAGAGGAAG	60				
3D	HJV-3FD	CCGGACCCTTGTGACTATGA	60	279	56	B	2
	HJV-3RD	GTGCCGTGGAAGAATCCTC	62				
4A	HJV-4FA	TCAAGGATTGAGGGCCATAG	61	300	56	B	2
	HJV-4RA	TGGATCTCCACATGGTTCC	60				
4B	HJV-4FB	GGTGGATAATCTTCCTGTAGC	62	288	56	B	2
	HJV-4RB	CGACGATTGCGCTCTGAT	60				
4C	HJV-4FC	GCTCTCCTTCTCCATCAAGG	65	256	58	B	2
	HJV-4RC	CTGAGCTGCCACGGTAAAGT	62				
4D	HJV-4FD	GGGCTTCCAGTGGAAGATGC	62	238	58	B	2
	HJV-4RD	CCCCTTACTGAATGCAAAGC	60				
4E	HJV-4FE	CATCTCTTCCCCTCAGATGC	65	300	56	B	2
	HJV-4RE	GATCCGGAATGCAGTAACCT	60				
4F	HJV-4FF	AAGCAGGGCCTAGGAGACAC	62	283	58	B	2
	HJV-4RF	TGCTTTCAGCTCTTGCCTCT	60				
4G	HJV-4FG	CTGCATTCCGGATCTCTGTG	62	291	56	B	2
	HJV-4RG	TTTTGAATCAAGAAAGCAGAACA	56				
4H	HJV-4FH	TGTGTGTGTAAGGTATGTTCTGC	61	261	58	B	2
	HJV-4RH	CTGATACTTCCGAGCCCTCTTTC	65				

Abbreviations: 5', 5-prime; 3', 3-prime; ºC, degrees Celsius; bp, base pair; F, forward primer; *HJV*, hemojuvelin gene; $MgCl_2$, magnesium chloride; mM, millimoles per liter; PCR, polymerase chain reaction; R, reverse primer; T_A, annealing temperature; T_M, melting temperature. $T_M = 2(nA+nT)+4(nG+nC)$. References: All *HJV* primers designed F Booley (unpublished data).

2.4.2 PCR amplification reactions and conditions

PCR amplification of the various exons of *HFE, HMOX1, SLC40A1, HAMP, CYBRD1* and *HJV* were performed in a GeneAmp® 2700 PCR system (Applied Biosystems) using intronic primers (Tables 2.1 – 2.6). The PCR reaction was performed in a volume of 25 µl, consisting of 50 ng DNA template, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP) (Fermentas), 10 pmol of each primer, 1 x Taq buffer (Fermentas), $MgCl_2$ (Fermentas) as indicated in Tables 2.1 – 2.6 and 0.5 U Taq polymerase (Fermentas). Three different PCR cycle programs, referred to as cycles A to C, were employed for the amplification of the various exons (see Tables 2.1 – 2.6) and are as follows:

Cycle A

An initial denaturation step at 95ºC for five minutes, followed by 35 cycles of denaturation at 95ºC for two minutes and annealing as specified (indicated as the T_A of each exon in Tables 2.2, 2.3 and 2.5) for two minutes. This was followed by a final extension step at 72ºC for ten minutes.

Cycle B

An initial denaturation step at 94ºC for five minutes, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing as specified (indicated as the T_A of each exon in Tables 2.4 and 2.6) for 30 seconds and elongation at 72ºC for 30 seconds. This was followed by a final extension step at 72ºC for ten minutes.

Cycle C

An initial denaturation step at 95ºC for two minutes, followed by 35 cycles of denaturation at 95ºC for 30 seconds, annealing as specified (indicated as the T_A of each exon in Tables 2.1 and 2.3) for 45 seconds and elongation at 72ºC for 30 seconds. This was followed by a final extension step at 72ºC for ten minutes.

2.5 Agarose gel electrophoresis

Successful amplification of PCR products was tested on a 2% (w/v) horizontal agarose gel [consisting of 4 g agarose in 200 ml 1 x TBE (90 mM Tris-HCl, 90 mM boric acid (H_3BO_3) and 0.1 mM EDTA, pH 8.0) and 0.01% (v/v) ethidium bromide (EtBr)]. The PCR product (5 µl) was mixed with an equal volume of cresol red loading buffer (2 mg/ml cresol red and 35% (w/v) sucrose) and loaded onto the gel. To establish whether the correct fragments were amplified a 100 bp ladder (Fermentas), used as a molecular size marker, was also loaded onto the gel. The PCR products were resolved at 120 V for one hour in 1 x TBE buffer solution. The DNA was visualised by ultraviolet light transillumination assisted by a GeneSnap MultiGenius Bio Imaging System (Syngene).

2.6 Heteroduplex-Single Strand Conformation Polymorphism (HEX-SSCP) analysis

HEX-SSCP analysis (Kotze et al. 1995) was performed on a 30 cm vertical gel apparatus. The PCR products of the various exons were resolved on a 12% polyacrylamide (PAA) gel supplemented with urea ($(NH_2)_2CO$) [gel consisting of 7.5% (w/v) urea, 1.5 x TBE (135 mM Tris-HCl, 135 mM H_3BO_3 and 2 mM EDTA, pH 8.0), 12% (w/v) PAA (1% C of a 40% stock (99 acrylamide (AA): 1 bisacrylamide (BAA)), 0.1% (w/v) ammonium persulphate (APS) and 0.01% (v/v) N, N, N', N'- tetramethylethylenediamine (TEMED)].

A 0.75 mm gel was cast and once polymerised, prepared for loading of the PCR products. Gels were placed in a Hoefer SE 660 electrophoresis tank filled with fresh 1 x TBE buffer (90 mM Tris-HCl, 90 mM H_3BO_3 and 0.1 mM EDTA, pH 8.0). The upper buffer chamber was filled with 1.5 x TBE buffer. An equal volume of bromophenol blue loading buffer [consisting of 95% (v/v) formamide (H_2NCHO, de-ionised), 20 mM EDTA (disodiumsalt), 0.05% (w/v) xylene cyanol ($C_{31}H_{28}N_2Na_4O_{13}S$) and 0.05% (w/v) bromophenol blue ($C_{19}H_{10}Br_4O_5S$)] was added to the PCR products; heat denatured for ten minutes at 95ºC and then kept on ice. Approximately 15 µl of the denatured PCR product was loaded onto the gel. Electrophoresis was performed at 350 V (4ºC) for 16 hours.

Following electrophoresis, the PAA gels were silver stained according to Beidler et al. (1992). The gels were carefully removed from the plates and placed in a plastic container filled with fixing solution [10% (v/v) EtOH, 0.5% (v/v) acetic acid (CH_3COOH)]. The gels were shaken using a Belly Dancer (Stovall Life Science) for ten minutes. The fixing solution was subsequently discarded and the gels rinsed twice for one minute with distilled water. After

this process, the gels were stained using a staining solution [0.1% (w/v) silver nitrate ($AgNO_3$)]. The gels were, once again, shaken for ten minutes using the Belly Dancer, whereafter the staining solution was discarded and the gels rinsed for five seconds with distilled water. The gels were subsequently covered with developing solution [1.5% (w/v) sodium hydroxide (NaOH), 0.4% (v/v) formaldehyde (HCHO)] and shaken on the Belly Dancer until the DNA bands were visible. The developing solution was discarded, the gels rinsed twice with distilled water and stored between transparencies for preservation.

2.7 Restriction Fragment Length Polymorphism (RFLP) analysis

Exon 2 of the *HFE* gene containing the variant IVS2+4T→C was subjected to RFLP analysis using the *Rsa I* enzyme (recognition site: GT↓AC ; Fermentas Life Sciences). As this variant creates an *Rsa I* restriction site, the wild-type DNA generates a 257 bp PCR product. Digestion of the mutated DNA produces three fragments of 257 bp, 181 bp and 76 bp in the heterozygous state, while it produces two fragments of 181 bp and 76 bp in the homozygous state.

To allow a more accurate discrimination between the different genotypes of the Q248H polymorphism, exon 6 of the *SLC40A1* gene was subjected to RFLP analysis, using the restriction endonuclease *Pvu II* (recognition site: CAG↓CTG; Fermentas Life Sciences). As the Q248H polymorphism abolishes a *Pvu II* restriction site, the digested 368 bp PCR product produces two fragments of 299 bp and 69 bp in wild-type DNA. In mutated DNA, digestion produces three fragments of 368 bp, 299 bp and 69 bp in the heterozygous state and one fragment of 368 bp is generated in the homozygous state.

PCR amplification of *SLC40A1* exon 6 for the individual fragments were performed as indicated in section 2.3.2. The PCR products subjected to RFLP analysis with the respective enzymes were digested overnight in 20 µl reactions consisting of 10 µl PCR product, 2 U of the relevant enzyme and 1 X buffer, in a 37ºC water bath.

Electrophoresis of the digested PCR products of *HFE* and *SLC40A1* was performed on a 2% (w/v) horizontal agarose gel [consisting of 4 g agarose in 200 ml 1 X TBE (90 mM Tris-HCl, 90 mM boric acid and 1 mM EDTA, pH 8.0)]. Loading of the digested PCR product, electrophoresis and visualisation are as described in section 2.4.

2.8 Semi-automated DNA sequencing

DNA purification

Prior to semi-automated DNA sequencing, PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). Briefly, one SV Column was placed into a collection tube for purification of the PCR product. An equal volume of membrane binding solution (4500 mM guanidine isothiocyanate, 500 mM potassium acetate, pH 5.0) was added to the PCR product and the mixture was incubated at room temperature for one minute. This was followed by centrifugation at 16000 x *g* for one minute. The flowthrough in the collection tube was subsequently discarded and the minicolumn was re-inserted into the collection tube.

Prior to its use, the membrane wash solution was diluted with 95% (v/v) EtOH. To wash the column, 700 µl of membrane wash solution (10 mM potassium acetate, 80% (v/v) ethanol,

16.7 mM EDTA, pH 8.0) was added to the assembly. The assembly was once again centrifuged at 16000 x *g* for one minute, where after the flowthrough was discarded. The column was rewashed with 500 µl membrane wash solution and centrifuged at 16000 x *g* for five minutes. The collection tube was emptied and the assembly re-centrifuged for one minute with the microcentrifuge lid open (or off), to allow the evaporation of any residual ethanol.

The minicolumn was carefully transferred to a 1.5 ml microcentrifuge tube and 50 µl of nuclease-free water was then added directly onto the center of the minicolumn. Following a one minute incubation step at room temperature, the column was centrifuged at 16000 x *g* for one minute. The minicolumn was discarded and the DNA subsequently stored at 4ºC.

Semi-automated sequencing

Semi-automated DNA sequencing was performed on PCR products demonstrating variation with HEX-SSCP analysis. The primers used for sequencing are the same as those used for PCR amplification (Tables 2.1 – 2.6). Cycle sequencing reactions consisted of 9.9 ng purified PCR product, 1 µl ready reaction mix [BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems)] and 3.3 pmol primer. The reaction was performed in a GeneAmp® PCR System 2700 (Applied Biosystems). The cycle sequencing program used was as follows: an initial denaturation step at 96ºC for ten seconds, followed by 25 cycles of denaturation at 96ºC for ten seconds, annealing at 55ºC for ten seconds and an extension step at 60ºC for four minutes. Semi-automated DNA sequencing was performed on an ABI Prism 3130XL Genetic Analyser (Applied Biosystems).

DNA sequences and chromatograms were compared and analysed using the BioEdit Sequence Alignment Editor V6.07 (Hall 1999) and Chromas 2.13 (http://technelysium.com.au/chromas.html).

2.9 Statistical analysis

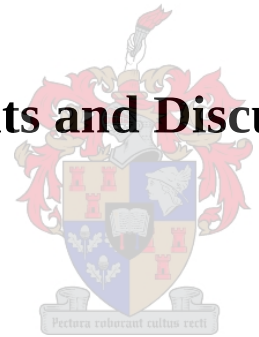
Allele and genotype frequencies were estimated by allele counting. Statistical differences between patient and control groups were tested by the Fisher's Exact Test and/or chi-squared (χ^2) analysis. Probability values smaller than 0.05 were regarded as statistically significant. The Hardy-Weinberg equilibrium (HWE) was performed to test equilibrium for the genetic traits investigated. Analyses were performed using the Microsoft Excel 2000 software and all results were verified with both STATISTICA [StatSoft, Inc. (2003) STAT (data analysis software system), version 6] and Epi Info [Epi Info™ (utilities StatCalc) v3.3.2, release date: 9 February 2005; Division of Public Health Surveillance] computer software. The frequencies of combined mutation carriers in patient and control subjects were compared to elucidate possible gene-gene interaction, according to Butt et al. (2003).

Table 2.7. List of generally used chemicals/reagents and their suppliers

Chemical/Reagent	Supplier
Acetic acid	Associated Chemical Enterprises
Acrylamide	Fluka
Agarose	BIO BASIC
Ammonium chloride	Merck
Ammonium persulphate	Associated Chemical Enterprises
Bisacrylamide	Sigma
Boric acid	Kimix
Bromophenol blue	Merck
Cresol red	Merck
EDTA	Merck
Ethanol	Kimix
Ethidium bromide	Fluka
Formaldehyde	Associated Chemical Enterprises
Formamide (de-ionised)	Merck
Phosphate buffered saline	Roche Diagnostics
Potassium chloride	Merck
Potassium hydrogen carbonate	Merck
Proteinase K	Roche Diagnostics
Silver nitrate	Merck
Sodium chloride	Sigma
Sodium dodecyl sulphate	Merck
Sodium hydroxide	Merck
Sucrose	Associated Chemical Enterprises
TEMED	Fluka
Tris-HCl	Fluka
Urea	Sigma
Xylene cyanol	Fluka

Chapter 3

Results and Discussion



Presented in the form of a full-length manuscript in preparation for future publication in a scientific journal

Mutation analysis of genes involved in iron homeostasis in Black South African patients with squamous cell carcinoma of the oesophagus

Abstract

OC is a disease characterised by a disproportionate presentation in certain ethnic groups, with squamous cell carcinoma (SCC) occurring more often in Blacks and adenocarcinoma (ADC) being more prevalent in Caucasians. Several factors have been attributed to the development of OC, including an excess of iron (leading to enhanced tumour growth), oesophageal injury and chronic inflammation. The purpose of this study was to assess the likelihood that variations identified in genes implicated in iron homeostasis (including *HFE*, *HMOX1*, *SLC40A1*, *HAMP*, *CYBRD1* and *HJV*), contribute to OC susceptibility. The study population included 50 unrelated OC patients with SCC and 50 population-matched control individuals of the Black Xhosa-speaking South African population. PCR amplification was performed on these genes, followed by heteroduplex single strand conformational polymorphism (HEX-SSCP) analysis. Twenty-one previously described and thirteen novel variants (***HFE***: Y342; ***HMOX1***: G255R, R262H, R262C; ***SLC40A1***: IVS5-27A→C, L378, 3'UTR+284C→T, 3'UTR+289G→A, 3'UTR+289G→T; ***CYBRD1***: L17, P195; ***HJV***: 5'UTR-1401T→C, 3'UTR+47A→G) were identified using semi-automated DNA sequencing analysis. Several of the variants identified in this study have previously been associated with iron overload and disease development in the Coloured and Caucasian populations of South Africa. This study identified several variants that could potentially contribute to disease susceptibility and is the first study demonstrating the potential involvement of iron dysregulation in OC susceptibility in the Black South African population.

Introduction

Oesophageal cancer (OC) is the eighth most common cancer worldwide (Parkin et al. 2005). The disease exhibits a distinct geographic variation in incidence as it occurs at high frequencies in certain defined global regions (Blot 1994, Parkin et al. 2005). The highest incidence of OC in South Africa has been identified in the Transkei region. SCC is the most common cancer among Black males [age standardised incidence rate (ASIR) 13.6/100 000] and the third most common cancer among Black females [ASIR 5.8/100 000] (Sitas et al. 1998) in this country.

Numerous risk factors for SCC have been identified, with the aetiological importance of the synergistic use of tobacco and alcohol being consistently displayed in SCC studies in Western countries (Blot 1994, Parkin 2001). Risk factors contributing to a lesser extent to the development of SCC include nutrition, nitrosamines, mycotoxins, infectious agents, consumption of hot beverages, repeated oesophageal injury, chronic inflammation and occupational exposures (Van Rensburg 1981, Syrjanen 1982, Norell et al. 1983, Hille et al. 1986, IARC 1986, 1988, Cheng et al. 1992, Gustavsson et al. 1993, Yang and Wang 1993, Blot 1994, Klimstra 1994, Parent et al. 2000). OC is known for its aggressive clinical behaviour and poor prognosis and develops in mid to late adulthood, rarely in individuals younger than 25 years.

Evidence for the involvement of iron in cancer was illustrated where OC developed as a consequence of iron supplementation in a rat model (Goldstein et al. 1998, Chen et al. 1999, 2000). Previously, it had been believed that iron overload in the Black South African population resulted from the excessive consumption of home-brewed alcoholic beverages that

had been contaminated with iron (MacPhail et al. 1979). Iron overload, however, does not develop in all beer drinkers and not all patients suffering from iron overload consume excessive amounts of beer. An excessive dietary intake of iron has also been linked to the pathogenesis of hepatocellular carcinoma in the Black South African population (Mandishona et al. 1998). Iron overload has been reported as a risk factor in the development of OC in several other populations, including a Danish population with primary haemochromatosis, where an increased risk for OC development was illustrated (Hsing et al. 1995). It has also been demonstrated that in conditions of iron overload, the cytotoxic activity of the macrophages is impaired, subsequently favouring tumour growth (Green et al. 1988, Huot et al. 1990).

To investigate iron as a possible risk factor in the development of oesophageal cancer, this study focused on the analysis of genes involved in iron homeostasis (including *HFE, HMOX1, SLC40A1, HAMP, CYBRD1* and *HJV*) in patients diagnosed with SCC. The HFE protein binds to the transferrin receptor (TFR), thereby reducing its affinity for transferrin (Feder et al. 1998). HMOX1 is vital in haem catabolism, degrading haem to biliverdin (Tenhunen et al. 1969). SLC40A1 is a highly conserved multiple transmembrane-domain protein involved in the export of iron from duodenal enterocytes and macrophages, mediating the efflux of iron in the presence of a ferroxidase (Abboud and Haile 2000, Donovan et al. 2000, McKie et al. 2000). HAMP has been identified as a key molecule in the regulation of iron homeostasis. CYBRD1 catalyses the reduction of ferric (Fe^{3+}) to ferrous iron (Fe^{2+}) in the gut lumen during intestinal iron absorption (McKie et al. 2001), with HJV a possible modulator of *HAMP* expression (Pantopoulos and Papanikolaou 2005).

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Materials and Methods

The study protocol was approved by the Ethics Review Committee of the University of Stellenbosch (Project number 2002/C071). Information regarding the study participants, the experimental procedures employed and the statistical analysis performed are as described in Chapter 2.

Results

The study population included 25 (50%) males and 25 (50%) females with a mean age of 59 years and 62 years, respectively (Table 3.1). The majority of patients (80% males and 76% females) were older than 50 years. Additional information was available for only 23 (92%) males and 19 (76%) females. Seven patients (males: 8.7%; females: 10.5%) never smoked and of these patients only one consumed alcoholic beverages. Of the 38 cigarette smokers, three (males: 2.6%; females: 5.3%) were only smokers, 19 (males: 31.6%; females: 18.48%) additionally consumed alcoholic beverages (beer and spirits), 15 (males: 21.1%; females: 18.4%) consumed only beer, and one (females: 2.6%) additionally consumed only spirits. Information regarding the occupations of the study participants was available for only 23 males and 19 females, with the majority of patients being unemployed, domestic workers, farm workers or gardeners. All of the OC patients were from the Eastern Cape region in South Africa.

Patient iron levels were also denoted, according to Section 2.2, Chapter 2. Three of 25 females were iron deficient (ferritin < 20µg/l), while nine females and six males had raised ferritin (with transferrin saturation < 45%). In addition, one female had raised ferritin (with

transferrin saturation > 45%), while only 12 females and 19 males had normal iron levels (transferrin saturation for all < 45%).

Table 3.1. Characteristics of the OC patients

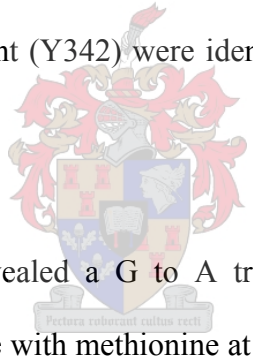
	Patients with OC	
	Males (n = 25)	**Females (n = 25)**
Average age (years)	59	62
Cigarette smoking	20 (80 %)	15 (60 %)
Never	3 (12 %)	4 (16 %)
Unknown	2 (8 %)	6 (24 %)
Alcoholic consumption (Beer)		
Weekly	12 (48 %)	8 (32 %)
Daily	1 (4 %)	3 (12 %)
Seldom	4 (16 %)	4 (16 %)
Never	4 (16 %)	4 (16 %)
Stopped	2 (8 %)	0
Unknown	2 (8 %)	6 (24 %)
Alcoholic consumption (Wine/Spirits)		
Weekly	8 (32 %)	2 (8 %)
Daily	0	1 (4 %)
Seldom	5 (20 %)	4 (16 %)
Never	10 (40 %)	12 (48 %)
Unknown	2 (8 %)	6 (24 %)

Mutation analysis

The variants identified in both patient and control groups, their allele frequencies as well as statistically significant associations between the patient and population-matched control groups, are shown in Table 3.2. All of the variants detected by HEX-SSCP analysis were verified by bi-directional sequencing.

HFE

Seven previously described (V53M, H63D, H63, IVS2+4T→C, IVS4-44T→C, C282Y and IVS5-47G→A) and one novel variant (Y342) were identified in the *HFE* gene (indicated in Table 3.2).



HEX-SSCP analysis of exon 2 revealed a G to A transition at nucleotide position 175, resulting in the replacement of valine with methionine at amino acid 53 (V53M). This variant presented only in the heterozygous state in a single (2%) OC patient and was also only observed in one (2%) of the population-matched control individuals. Another missense mutation identified in exon 2 was the C to G transversion at nucleotide position 187, causing the substitution of histidine with aspartic acid at amino acid 63 (H63D). This mutation was only detected in two of 50 (4%) of the OC patients, one being heterozygous and the other homozygous, and was absent from the population-matched control individuals.

Table 3.2. Variants identified in the Black South African population

Gene	Exon/ Intron	Variant	Allele frequencies[a]		*P*	Reference
			Patients	Controls		
HFE	2	V53M[b,d]	0.01	0.01	0.74	de Villiers et al. 1999
	2	H63D[c,d]	0.03	0.00	-	Feder et al. 1996
	2	H63[b,e]	0.00	0.02	-	de Villiers et al. 1998
	2	IVS2+4T→C[c]	0.39	0.42	0.72	Beutler and West 1997
	4	IVS4-44T→C[b]	0.05	0.06	0.76	Beutler and West 1997
	4	C282Y[b,d]	0.02	0.00	-	Feder et al. 1996
	5	IVS5-47G→A [b]	0.63	0.63	0.97	Beutler and West 1997
	6	Y342[b,e]	0.00	0.01	-	This study
HMOX1	2	IVS2-19C→T[c]	0.03	0.01	0.31	SeattleSNPs
	4	IVS4+51delTGGCTGTCTGACT[b]	0.06	0.07	0.77	Zaahl et al. 2005
	4	IVS4+59C→G[b,d]	0.02	0.00	-	SeattleSNPs
	5	G255R[b,d]	0.01	0.00	-	This study
	5	R262C[b,d]	0.01	0.00	-	This study
	5	R262H[b,d]	0.01	0.00	-	This study
SLC40A1	5'UTR	5'UTR-98G→C[b]	0.06	0.04	0.41	Douabin-Gicquel et al. 2001
	5'UTR	5'UTR-23A→G[b]	0.02	0.01	0.52	Zaahl et al. 2004
	5'UTR	5'UTR-8C→G[b,d]	0.01	0.00	-	Douabin-Gicquel et al. 2001
	2	IVS1-24G→C[c]	0.35	0.28	0.27	Devalia et al. 2002
	4	I109[c]	0.07	0.05	0.55	Beutler and West 2003
	4	L129[c]	0.07	0.10	0.45	Zaahl et al. 2004
	5	IVS5-27A→C[b,d]	0.03	0.00	-	This study
	6	V221[c]	0.18	0.14	0.48	Devalia et al. 2002
	6	Q248H[b]	0.08	0.09	0.87	Gordeuk et al. 2003
	7	L378[b,d]	0.01	0.00	-	This study
	3'UTR	3'UTR+284C→T[b]	0.03	0.01	0.31	This study
	3'UTR	3'UTR+289G→A[b]	0.02	0.03	0.50	This study
	3'UTR	3'UTR+289G→T[b]	0.05	0.04	0.50	This study
CYBRD1	1	L17[b]	0.02	0.01	0.53	This study
	1	IVS1-4C→G[b]	0.05	0.04	0.54	Zaahl et al. 2004
	2	IVS2+8T→C[c]	0.83	0.84	0.85	Zaahl et al. 2004
	4	P195[b,d]	0.03	0.00	-	This study
HJV	5'UTR	5'UTR-1401T→C[b]	0.03	0.01	0.30	This study
	4	A310G[b]	0.04	0.05	0.46	Lee et al. 2004
	3'UTR	3'UTR+47A→G[b]	0.05	0.04	0.50	This study

[a] Allele frequencies of only the polymorphic allele denoted; [b] variants identified only in the heterozygous state; [c] variants identified in both the heterozygous and homozygous states; [d] variants identified only in the patient group; [e] variants identified only in the control group. Abbreviations: *CYBRD1* , cytochrome b reductase 1 gene; *HFE* , high iron gene; *HJV* , hemojuvelin; *HMOX1* , haem oxygenase 1 gene; IVS, intervening sequence; *P* , probability; *SLC40A1* , solute carrier family 40 (iron regulated transporter) member 1 gene; UTR, untranslated region.

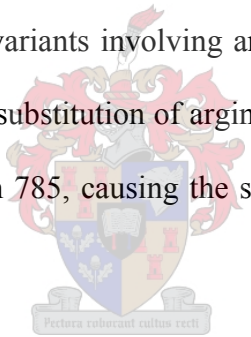
A previously described T to C transition was observed at nucleotide position 189, resulting in no substitution of amino acid 63 (H63); two nucleotides downstream of the H63D variant position. This synonymous variant was only identified in the heterozygous state in two of 46 (4.4%) population-matched control individuals and was absent from the OC patient group. HEX-SSCP analysis of exon 4 revealed a G to A transition at nucleotide position 845, resulting in a cysteine to tyrosine substitution at amino acid 282 (C282Y). This variant presented only in the heterozygous state in two of the 50 (4%) OC patients and did not occur in any of the population-matched control individuals.

One of the non-coding variants identified in this study, was a T to C transition (IVS2+4T→C) found in intron 2 of the *HFE* gene. This variant occurred at high frequencies in both the OC and control populations and was observed in both the heterozygous [25 of 50 (50%) OC patients and 21 of 47 (44.7%) population-matched control individuals] and homozygous states [seven of 50 (14%) OC patients and nine of 47 (19.2%) population-matched control individuals]. Another intronic variant involving a T to C transition in the *HFE* gene was IVS4-44T→C. This variant was restricted to the heterozygous state in the OC patient group, with five of the 50 (10%) OC patients being heterozygous. Screening of the population-matched control individuals revealed six of 48 (12.5%) heterozygous and a single (2.1%) homozygous individual. The G to A transition in intron 5 (IVS5-47G→A) was observed in both the heterozygous [26 of 49 (53.1%) OC patients and 19 of 50 (38%) population-matched control individuals] and the homozygous states [18 of 49 (36.7%) OC patients and 23 of 50 (46%) population-matched control individuals].

HMOX1

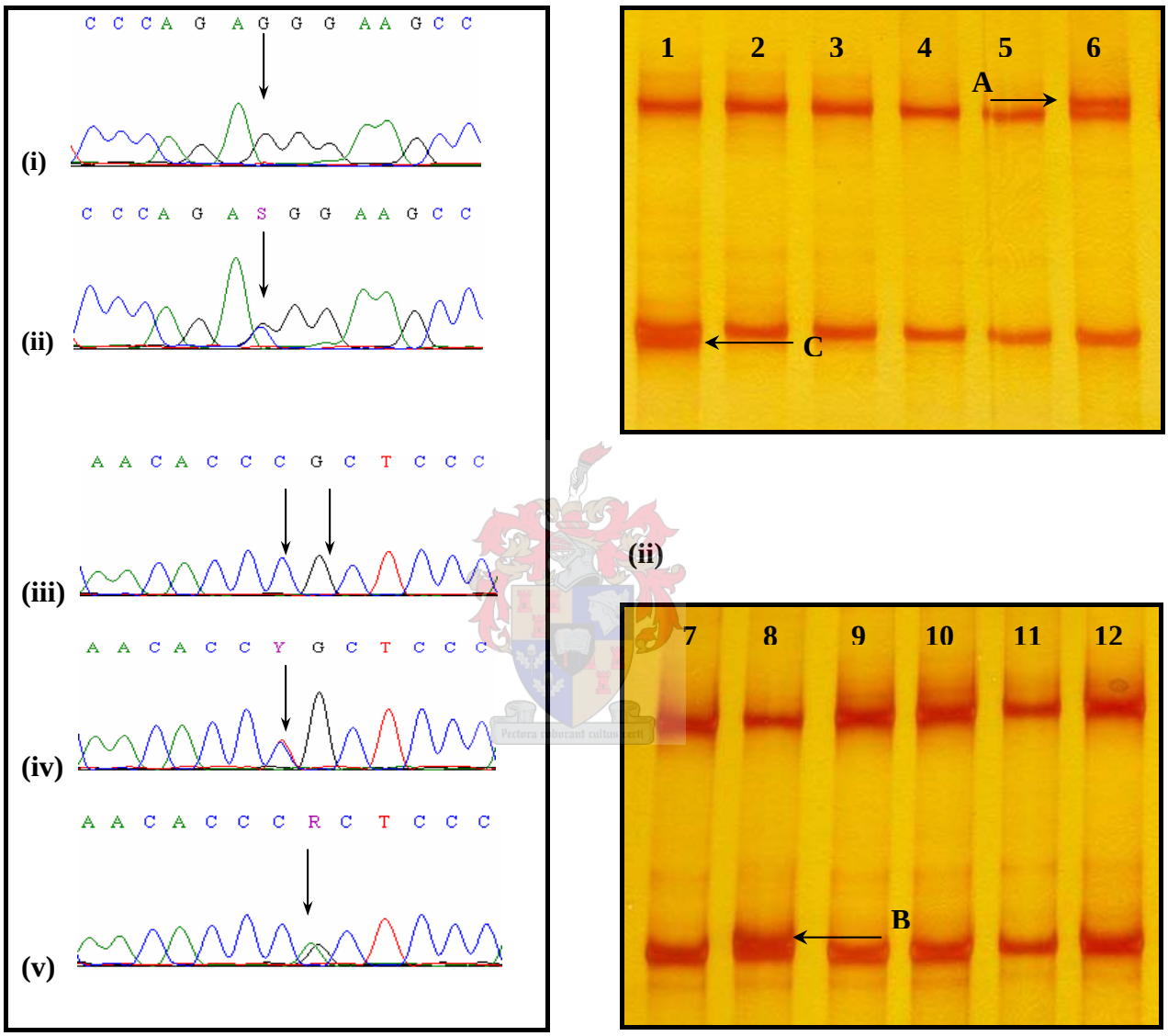
Mutation analysis of the *HMOX1* gene using a combined HEX-SSCP method revealed three previously described (IVS2-19C→T, IVS4+51delTGGCTGTCTGACT and IVS4+59C→G) and three novel (G255R, R262C and R262H) variants (indicated in Table 3.2).

The three novel variants identified in this gene, were all discovered in exon 5 and are shown in Fig. 3.1. All three variants were limited to the heterozygous state and each of these was observed in a single OC individual. The first variant is a G to C transversion at nucleotide position 763, resulting in a glycine to tyrosine substitution at amino acid 255 (G255R). Two OC patients were heterozygous for variants involving amino acid 262, a C to T transition at nucleotide position 784, causing the substitution of arginine with cysteine (R262C) and the G to A transition at nucleotide position 785, causing the substitution of arginine with histidine (R262H).



The first intronic variant detected in the *HMOX1* gene, was a C to T transition located 19 nucleotides upstream of exon 3 (IVS2-19C→T). The heterozygous state of the variant was detected in one of 50 (2%) OC patients and in one of 50 (2%) of the population-matched control individuals. The homozygous state of IVS2-19C→T was limited to one individual in the OC patient group. IVS4+51delTGGCTGTCTGACT was restricted to the heterozygous state and was identified in six of 50 (12%) of the OC patients and seven of 50 (14%) of the population-matched control individuals. The previously described C to G transversion, IVS4+59C→G, was detected only in the heterozygous state in two of 50 (4%) of the OC patients. This variant did not occur in any of the population-matched control individuals.

Figure 3.1. Schematic representation of the novel variants identified in exon 5 of the *HMOX1* gene.



Legend to Figure 3.1

A. Sequencing electropherograms indicating: (i) and (iii) the wild-type sequence, (ii) the variant G255R (G→C), (iv) the variant R262C (C→T) and (v) the variant R262H (G→A). Arrows indicate the point of variation, red, thymine (**T**); blue, cytosine (**C**); green, adenine (**A**); black, guanine (**G**). **B.** HEX-SSCP gel stained with $AgNO_3$, with the wild-type banding patterns in lanes 2-5, 7 and 9-12. Lanes 1, 6 and 8 contain DNA of individuals heterozygous for the respective variants (A) G255R, (B) R262C and (C) R262H.

SLC40A1

Eight previously described (5'UTR-98G→C, 5'UTR-23A→G, 5'UTR-8C→G, IVS1-24G→C, I109, L129, V221 and Q248H) and four novel variants (IVS5-27A→C, 3'UTR+284C→T, 3'UTR+289G→A and 3'UTR+289G→T) were identified in *SLC40A1* (indicated in Table 3.2).

Three variants were observed in the 5' UTR flanking the iron-responsive element (IRE): a G to C transversion at nucleotide position –98 (5'UTR-98G→C), an A to G transition at nucleotide position –23 (5'UTR-23A→G) and a C to G transversion at nucleotide position –8 (5'UTR-8C→G), relative to the initiating ATG. All three of these variants were limited to the heterozygous state. The 5'UTR-8C→G variant was found in a single (2%) OC patient and in none of the population-matched control individuals, while 5'UTR-23A→G occurred in two of 50 (4%) of the OC patients and in one of 47 (2.1%) population-matched control individuals. The last variant, 5'UTR-98G→C, was identified in six of 50 (12%) OC patients and in four of 47 (8.5%) population-matched control individuals.

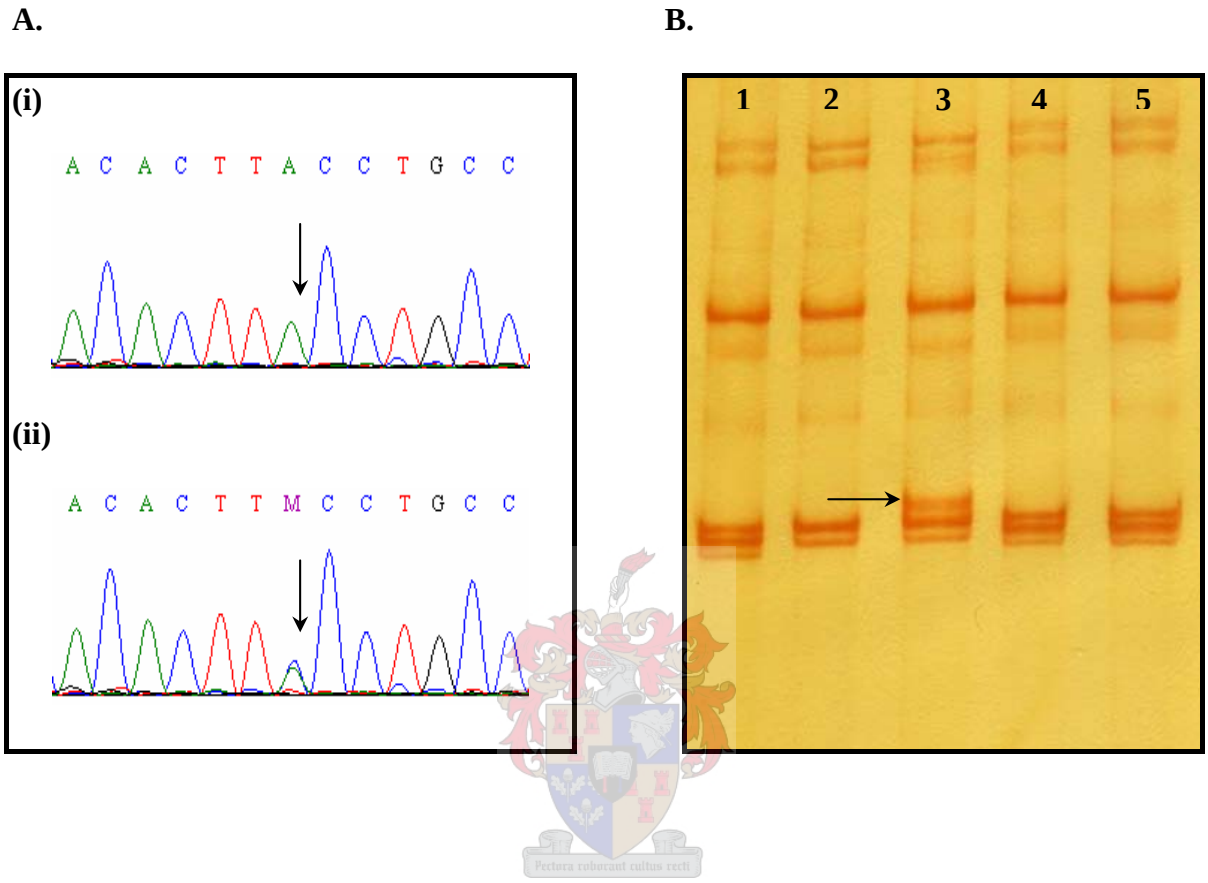
Five exonic variants were identified in the *SLC40A1* gene. A previously described C to T transition was observed at nucleotide position 327, resulting in no substitution of amino acid 109 (I109) in exon 4. This synonymous variant was identified in the heterozygous state [five of 48 (10.4%) OC patients and five of 50 (10%) population-matched control individuals], with only one (2.1%) of the OC individuals being homozygous. Another C to T transition was observed at nucleotide position 387, resulting in no substitution of amino acid 129 (L129) in exon 4. This variant was identified in both the heterozygous [five of 48 (10.4%) OC patients and eight of 50 (16%) population-matched control individuals] and homozygous

states [one of 48 (2.1%) OC patients and one of 50 (2%) population-matched control individuals].

The only previously described synonymous variant detected in exon 6 of *SLC40A1*, is a T to C transition at nucleotide position 662, resulting in no substitution of amino acid 221 (V221). Both the heterozygous [11 of 48 OC (22.9%) patients and 12 of 50 (24%) controls] and the homozygous states [three of 48 (6.3%) OC patients and none of the controls] of this variant were revealed. The G to T transversion at nucleotide position 744 of the *SLC40A1* gene resulted in the replacement of glutamine with histidine at amino acid 248 (Q248H). This polymorphism was detected in exon 6 of both the OC and control individuals, but was found only in the heterozygous state in eight of 48 (16.7%) OC patients and six of 50 (12%) control individuals. The only novel exonic variant detected in *SLC40A1*, was a T to C transition at nucleotide position 1132, resulting in no substitution of amino acid 378 (L378) in exon 7. This novel synonymous variant, indicated in Fig. 3.3, was detected in a single OC patient and was restricted to the heterozygous state. The variant did not occur in any of the population-matched control individuals.

The first intronic variant observed in the *SLC40A1* gene, was a G to C transversion in intron one (IVS1-24G→C). The heterozygous variant presented in 25 of 47 (53.2%) OC patients and in 18 of 47 (38.3%) of the population-matched control individuals. Four (8.5%) of the OC patients, as well as four (8.5%) of the control individuals were homozygous for IVS1-24G→C. HEX-SSCP analysis revealed a novel A to C transversion in intron five (IVS5-27A→C) of the gene, indicated in Fig 3.2. This variant occurred only in the heterozygous state in a single (2.1%) OC patient and in two (4%) of the population-matched controls.

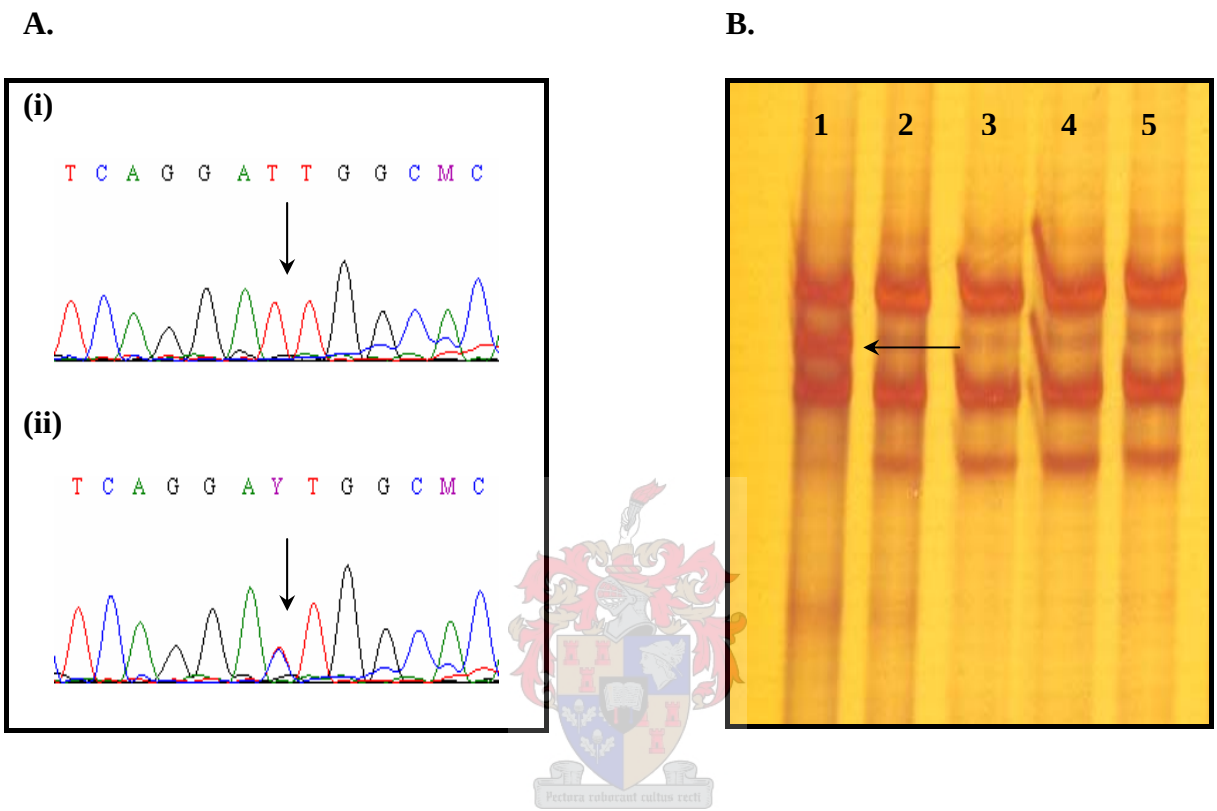
Figure 3.2. Schematic representation of the novel variant identified in intron 5 of the *SLC40A1* gene.



Legend to Figure 3.2

A. Sequencing electropherograms indicating: (i) the wild-type sequence and (ii) the variant IVS5-27A→C. Arrows indicate the point of variation, red, thymine (**T**); blue, cytosine (**C**); green, adenine (**A**); black, guanine (**G**). **B.** HEX-SSCP gel stained with $AgNO_3$, with the wild-type banding pattern in lanes 2 and a previously described variant (V221) in lanes 1, 4 and 5. Lane 3 contains DNA of an individual heterozygous for the variant IVS5-27A→C.

Figure 3.3. Schematic representation of the novel variant identified in exon 7 of the *SLC40A1* gene.



Legend to Figure 3.3

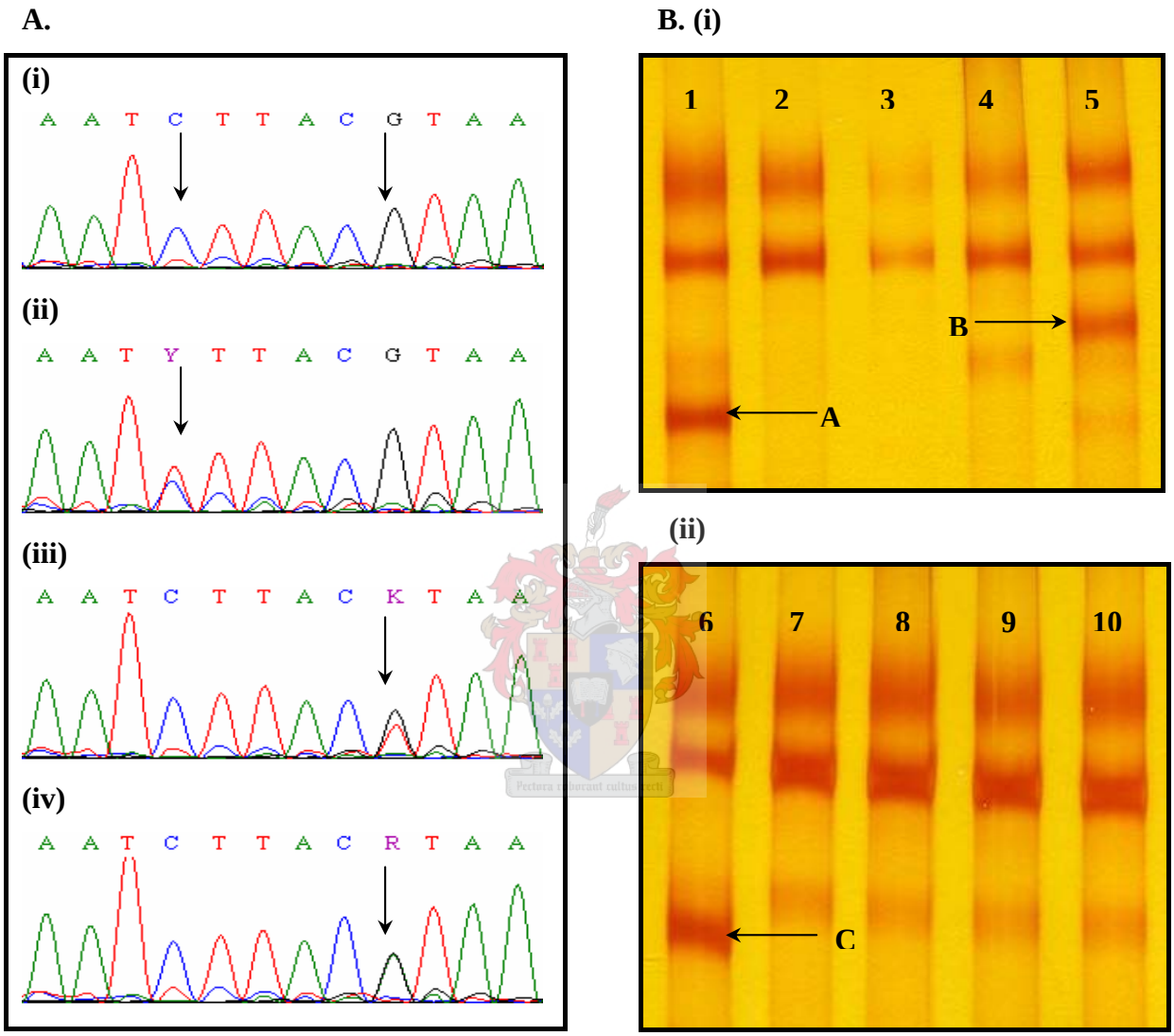
A. Sequencing electropherograms indicating: (i) the wild-type sequence and (ii) the variant L378 (T→C). Arrows indicate the point of variation, red, thymine (**T**); blue, cytosine (**C**); green, adenine (**A**); black, guanine (**G**). **B.** HEX-SSCP gel stained with $AgNO_3$, with the wild-type banding patterns in lanes 2, 3, 4 and 5. Lane 1 contains DNA of an individual heterozygous for the variant L378.

Three novel variants were identified in the 3'UTR of the *SLC40A1* gene, shown in Fig. 3.4. The first variant involved a C to T transition (3'UTR+284C→T) that was only found in the heterozygous state in three of 50 (6%) patients and in one of 50 (2%) control individuals. The other two variants both involved the same nucleotide. The first one involved a G to A transition (3'UTR+289G→A), observed only in the heterozygous state in two of 50 (4%) of the OC patients and three of 50 (6%) population-matched control individuals. The second variant involved a G to T transversion (3'UTR+289G→T) also limited to the heterozygous state, in five of 50 (10%) of the OC patients and four of 50 (8%) control individuals.

HAMP

No variants were detected in the *HAMP* gene using HEX-SSCP analysis.

Figure 3.4. Schematic representation of the novel variants identified in the 3'UTR of the *SLC40A1* gene.



Legend to Figure 3.4

A. Sequencing electropherograms indicating: (i) the wild-type sequence, (ii) the variant 3'UTR+284C→T, (iii) the variant 3'UTR+289G→T and (iv) the variant 3'UTR+289G→A. Arrows indicate the point of variation, red, thymine (**T**); blue, cytosine (**C**); green, adenine (**A**); black, guanine (**G**). **B.** HEX-SSCP gels stained with $AgNO_3$, with the wild-type banding patterns in lanes 2-5 and 7-9. Lanes 1, 6 and 10 contain DNA of individuals heterozygous for the respective variants (A) 3'UTR+284C→T, (B) 3'UTR+289G→T and (C) 3'UTR+289G→A.

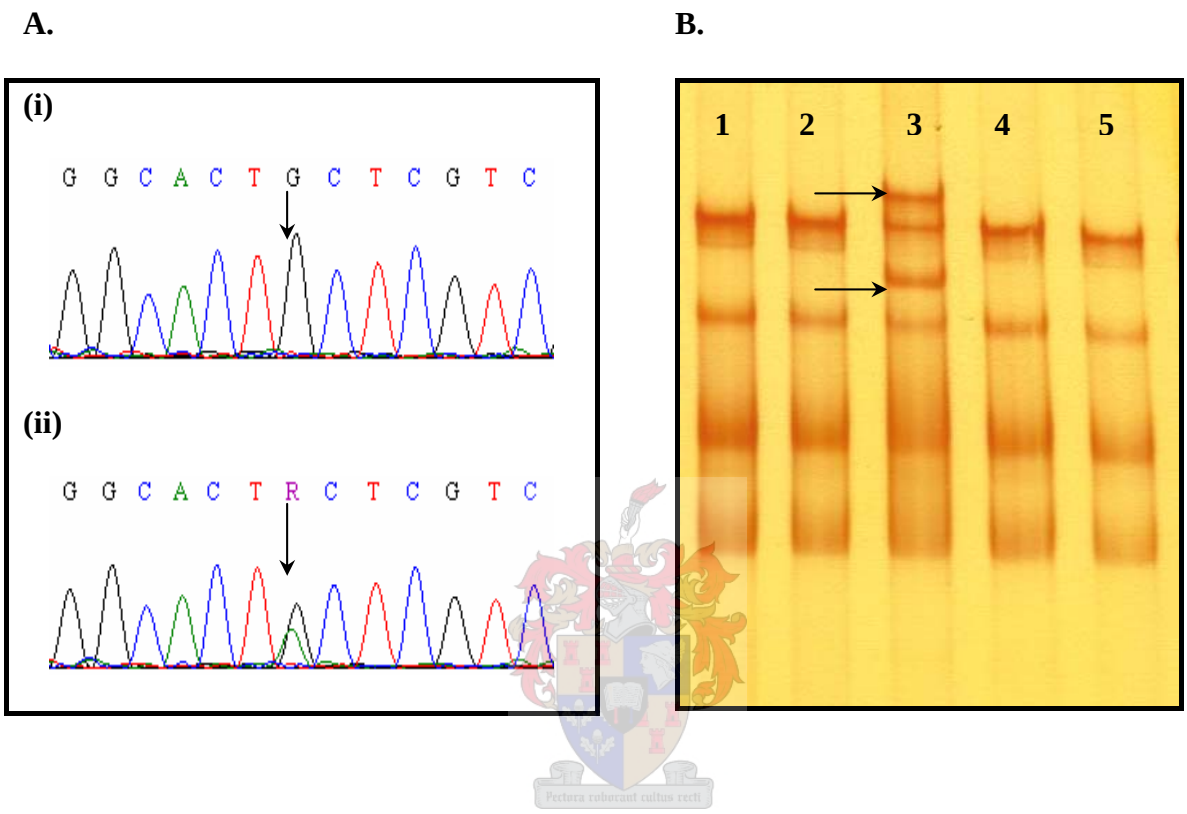
CYBRD1

Two previously described (IVS1-4C→G and IVS2+4T→C) and two novel synonymous variants (L17 and P195) were detected in the *CYBRD1* gene (indicated in Table 3.2).

The only variant observed in exon 1 of the *CYBRD1* gene, was a G to A transition at nucleotide position 51, resulting in no substitution of amino acid 17 (L17). This variant was restricted to the heterozygous state, with two of 48 (4.2%) of the OC patients and only a single (2.3%) control individual being identified. The G to A transition at nucleotide position 584, resulting in no substitution of amino acid 195 (P195) in exon 4, was identified in the heterozygous state in three of 48 (6.3%) of the OC patients. This variant was not identified in any of the population-matched control individuals.

Two intronic variants were identified in the *CYBRD1* gene. The first was a C to G transversion in intron one (IVS1-4C→G), detected only in the heterozygous state in five of 50 (10%) OC patients and in four of 47 (8.5%) of the population-matched control individuals. The second non-coding variant was a T to C transition detected in intron two (IVS2+8T→C). This variant presented in both the heterozygous [13 of 50 (26%) patients and 11 of 47 (23.4%) population-matched control individuals] and the homozygous states [35 of 50 (70%) patients and 34 of 47 (68%) population-matched control individuals].

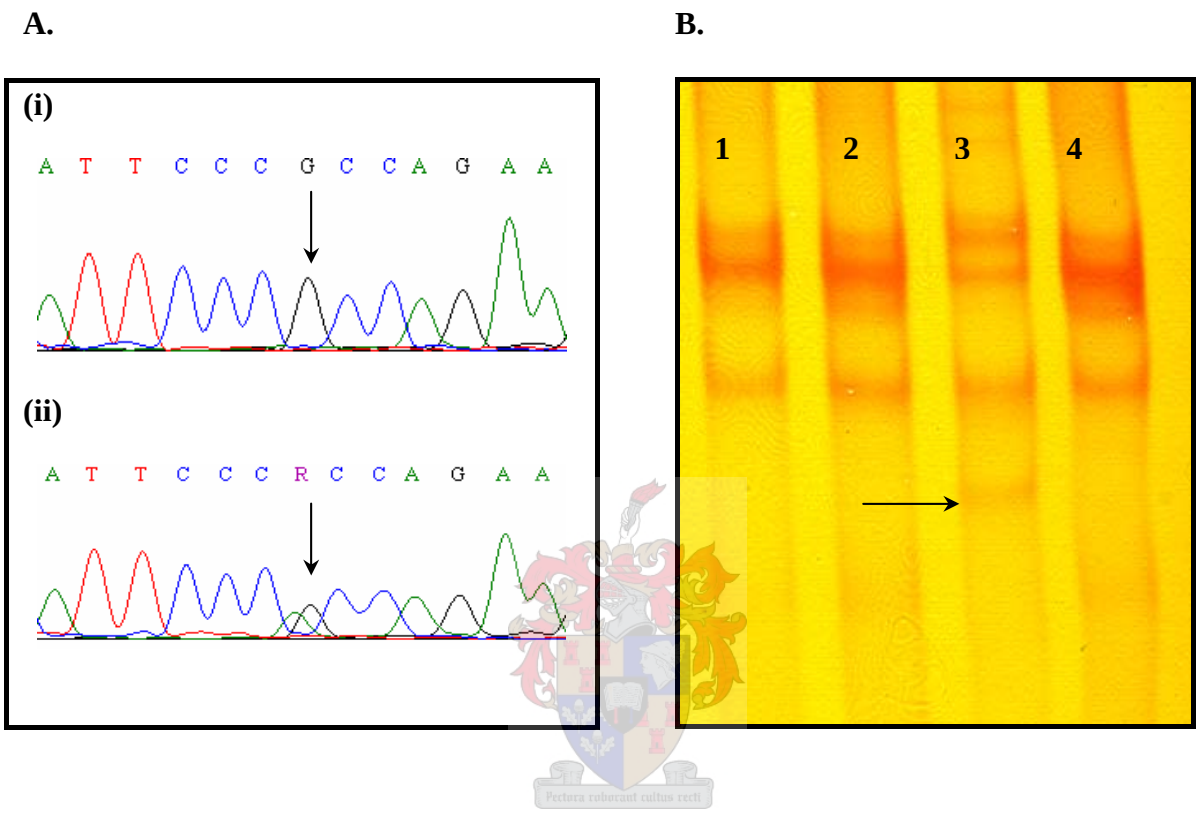
Figure 3.5. Schematic representation of the novel variant identified in exon 1 of the *CYBRD1* gene.



Legend to Figure 3.5

A. Sequencing electropherograms indicating: (i) the wild-type sequence and (ii) the variant L17 (G→A). Arrows indicate the point of variation, red, thymine (**T**); blue, cytosine (**C**); green, adenine (**A**); black, guanine (**G**). **B.** HEX-SSCP gel stained with $AgNO_3$, with the wild-type banding patterns in lanes 1, 2, 4 and 5. Lane 3 contains DNA of an individual heterozygous for the variant L17.

Figure 3.6. Schematic representation of the novel variants identified in exon 4 of the *CYBRD1* gene.



Legend to Figure 3.6

A. Sequencing electropherograms indicating: (i) the wild-type sequence and (ii) the variant P195 (G→A). Arrows indicate the point of variation, red, thymine (**T**); blue, cytosine (**C**); green, adenine (**A**); black, guanine (**G**). **B.** HEX-SSCP gel stained with $AgNO_3$, with the wild-type banding patterns in lanes 1, 2 and 4. Lane 3 contains DNA of an individual heterozygous for the variant P195.

HJV

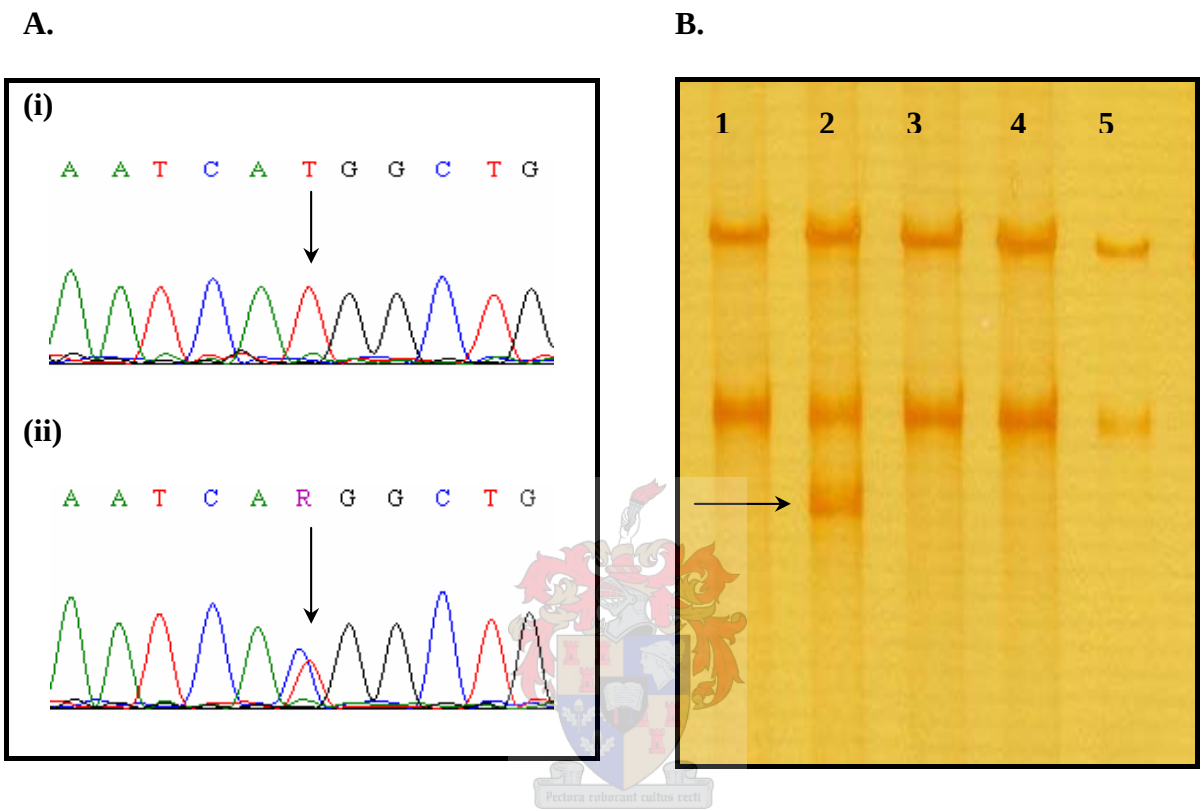
One previously described (A310G) and two novel variants (5'UTR-1401T→C and 3'UTR+47A→G) were detected in *HJV* (indicated in Table 3.2).

A novel T to C transition was observed in the 5'UTR at nucleotide position –1401 (5'UTR-1401T→C) relative to the initiating ATG (shown in Fig. 3.7). This variant was identified only in the heterozygous state in three of 50 (6%) of the OC patients and a single population-matched control individual.

The only exonic variant detected in *HJV*, was a previously described alanine to glycine change in exon 4 at amino acid position 310 (A310G). This C to G transversion at nucleotide position 929 was limited to the heterozygous state in four of 49 (8.2%) of the OC individuals and in five of 46 (10.9%) of the population-matched control individuals.

HEX-SSCP analysis revealed a novel A to G transition in the 3'UTR (3'UTR+47A→G) of the *HJV* gene (shown in Fig. 3.8). Five of 49 (10.2%) OC patients and four of 50 (8%) population-matched control individuals were heterozygous for this variant, while no homozygous individuals were observed in either of the groups. No variants were detected in exons 2 and 3 of the *HJV* gene using HEX-SSCP analysis.

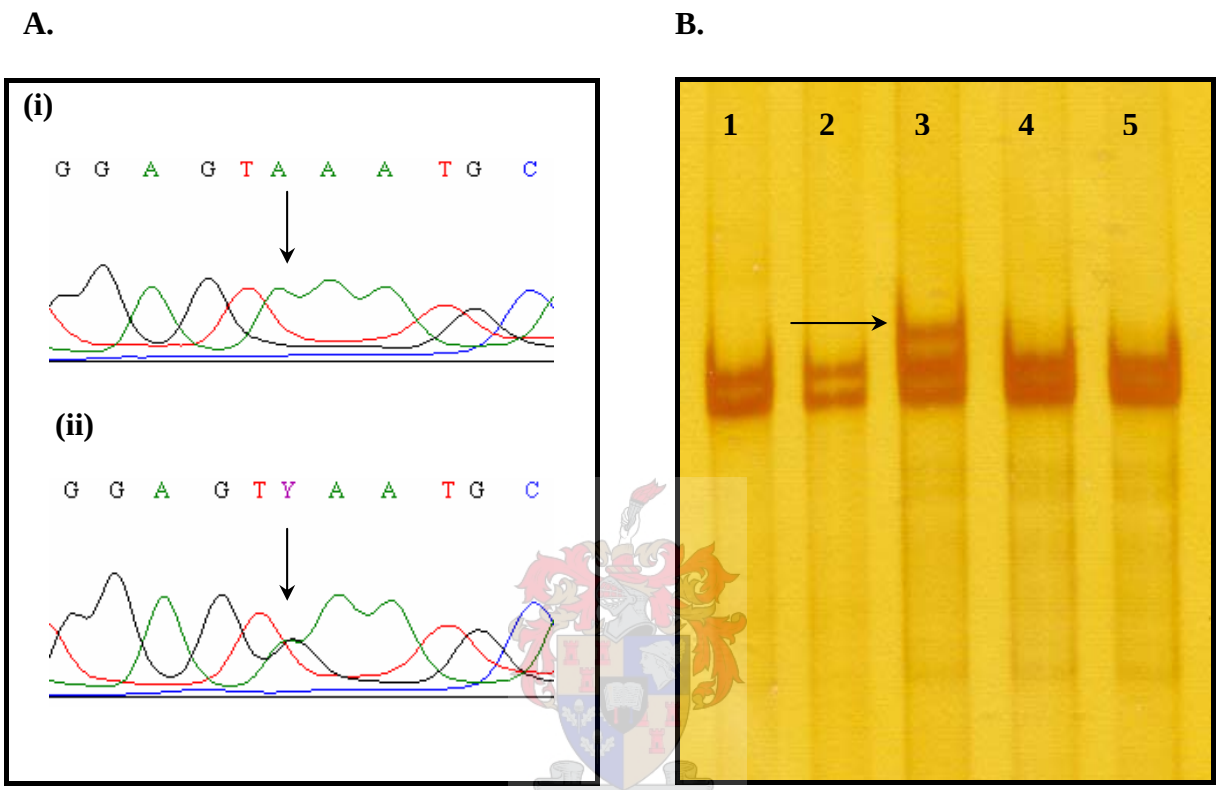
Figure 3.7. Schematic representation of the novel variant identified in the 5'UTR of the *HJV* gene.



Legend to Figure 3.7

A. Sequencing electropherograms indicating: (i) the wild-type sequence and (ii) the variant 5'UTR-1401 T→C. Arrows indicate the point of variation, red, thymine (**T**); blue, cytosine (**C**); green, adenine (**A**); black, guanine (**G**). **B.** HEX-SSCP gel stained with $AgNO_3$, with the wild-type banding patterns in lanes 1, 3, 4 and 5. Lane 2 contains DNA of an individual heterozygous for the variant 5'UTR-1401 T→C.

Figure 3.8. Schematic representation of the novel variant identified in the 3'UTR of the *HJV* gene.



Legend to Figure 3.8

A. Sequencing electropherograms indicating: (i) the wild-type sequence and (ii) the variant 3'UTR+47 A→G. Arrows indicate the point of variation, red, thymine (**T**); blue, cytosine (**C**); green, adenine (**A**); black, guanine (**G**). **B.** HEX-SSCP gel stained with $AgNO_3$, with the wild-type banding patterns in lanes 1, 2, 4 and 5. Lane 3 contains DNA of an individual heterozygous for the variant 3'UTR+47 A→G.

Discussion

Single-nucleotide polymorphisms (SNPs) can be employed as markers in the search to identify genes conferring susceptibility to common diseases (Kruglyak 1999). Approximately 90% of sequence variations can be attributed to SNPs. These SNPs occur at an estimated frequency of one per 1000 bases (Taillon-Miller et al. 1998).

A small number of studies investigating iron dysregulation in the Black South African population have been described in the literature. For this reason, where possible, the allele/genotype frequencies of variants detected in this study were compared to data available in the HapMap project, as well as other studies investigating these genes in the African and/or African-American populations. A few of the variants previously identified only in African and African-American individuals have also been observed in the Yoruba population of Nigeria that is included in the HapMap project (www.hapmap.org) as a representation of the African population.

5' UTR variants

Three previously described (***SLC40A1***: 5'UTR-98G→C, 5'UTR-23A→G and 5'UTR-8C→G) and one novel (***HJV***: 5'UTR-1401T→C) variant, have been identified in the 5' untranslated regions (UTRs) of the *SLC40A1* and *HJV* genes. The 5' UTRs of eukaryotic mRNAs are involved in numerous post-transcriptional regulatory pathways that control mRNA localisation, stability and translation efficiency (Sonenberg 1994). Iron-response elements have more recently been identified in the 5'UTRs of mRNAs with implications for

disease. Since many mRNAs that contain highly structured 5'UTRs are linked to growth control, it is not surprising that over-expression of components of the translation initiation mechanism is reportedly associated with tumorigenesis. Mutations in this region have been found to influence the translational efficiency of the mRNA (Pickering and Willis 2005). *SLC40A1* is the only gene investigated in this study with a known IRE in the 5'UTR and no IRE has been identified in the 5'UTR of the *HJV* gene. No literature has been identified indicating any possible functional effects of 5'UTR-98G→C, 5'UTR-23A→G and 5'UTR-8C→G on the IRE in the 5'UTR of *SLC40A1*.

All three previously described 5'UTR variants were identified in the study of Zaahl et al. (2004). Two of these variants, 5'UTR-98G→C and 5'UTR-8C→G, were only identified in the Black control population and in none of the iron overload patients. Both these variants were identified in the OC population of this study at a higher allele frequency than previously observed. This is possibly due to the small sample size (2n = 22) of the previous study and is not necessarily an indication that these variants contribute to OC susceptibility. 5'UTR-98G→C was identified in one male and five female OC patients. Three of these females demonstrated iron deficiency (serum ferritin < 20 μg/l), while the three remaining individuals had normal serum ferritin levels. It is possible that 5'UTR-98G→C, in combination with another variant, is responsible for the iron deficiency observed in three of the OC patients. Zaahl et al. (2004) identified 5'UTR-23A→G in eight of 11 Black HH patients and observed significant associations for this variant with iron overload. The study also observed this variant in *cis* with the $(CGG)_7$ allele found in the promoter region of *SLC40A1*. This variant was identified in one female with iron overload (serum ferritin = 424 μg/l) and one male with normal serum ferritin (213.1 μg/l). The 5'UTR-8C→G variant was identified in combination with 5'UTR-98G→C in one of the females with iron deficiency. The combined presence of

these two variants may have been responsible for the low ferritin levels observed. Although we did not detect any statistically significant associations in this study, it is possible that these variants may be in linkage disequilibrium with other disease-causing loci.

The novel variant 5'UTR-1401T→C was identified in the 5'UTR of the *HJV* gene of three OC patients, one female and two males with serum ferritin levels of 148 µg/l, 203.6 µg/l and 402.2 µg/l, respectively. This variant may be responsible for the high serum ferritin levels observed in these patients. However, functional studies have to be performed to determine the effect 5'UTR-1401T→C and the three known variants (***SLC40A1***: 5'UTR-98G→C, 5'UTR-23A→G, 5'UTR-8C→G) may have on gene expression.

Exonic variants

Nine previously described (***HFE***: V53M, H63D, H63, C282Y; ***SLC40A1***: I109, L129, V221, Q248H and ***HJV***: A310G) and seven novel (***HFE***: Y342; ***HMOX1***: G255R, R262C, R262H; ***SLC40A1***: L378 and ***CYBRD1***: L17, P195) exonic variants have been identified in this study.

The V53M variant identified in the *HFE* gene, involves an amino acid that has remained evolutionary conserved in human, mice and rat DNA. Valine and methionine are both non-polar amino acids that are highly hydrophobic. Methionine, however, contains sulphur that could create a disulfide bond with another peptide. The functional significance of this variant has not yet been determined. De Villiers et al. (1999) detected V53M in the Khoisan population as well as in three other black South African populations (Sotho/Pedi, Venda and Zulu) with iron overload. This variant has not been detected in any Caucasian individuals to

date. Functional studies need to be performed and larger population sizes should be screened to determine if this variant could possibly contribute to iron overload in Black populations.

Two major missense mutations of the *HFE* gene implicated in the development of primary iron overload, H63D and C282Y (Feder et al. 1996), were detected in this study. H63D has virtually no effect on the HFE protein structure (Dupradeau et al. 2000) and the homozygous H63D genotype is very rarely associated with iron loading (Hanson et al. 2001). C282Y modifies the folding of the HFE protein, subsequently impairing protein processing, transport, and cell surface expression. H63D requires the presence of the C282Y mutation to have a causative effect and it has been shown that individuals with excessive alcohol consumption in the presence of H63D are also susceptible to iron overload (Moirand et al. 1999). McNamara et al. (1998) have shown that the H63D and C282Y mutations in *HFE* are not responsible for African iron overload. Similar to H63D, C282Y is not as common amongst Black populations as in Caucasians and its presence in this study may be due to Caucasian admixture. It may be possible that the presence of these mutations together with other disease-causing variants in the Black population could contribute to the iron overload possibly associated with the development of OC.

Exonic splicing enhancers (ESEs) are *cis*-acting elements that function as binding sites for serine/arginine-rich (SR) proteins, a family of essential splicing factors that is also involved in alternative splicing regulation (Blencowe 2000). ESE-bound SR proteins promote splicing of adjacent introns. Each of the novel non-synonymous variants in this study was analysed using the ESE-prediction program ESEfinder (http://exon.cshl.edu/ESE) to determine if these variants may have an effect on splicing (Cartegni et al. 2003).

The G255R variant in *HMOX1* causes a change from glycine, a hydrophobic amino acid, to arginine, a polar amino acid. A homology search was conducted for exon 5 of the *HMOX1* gene using the BLAST program (http://www.ncbi.nlm.nih.gov/blast) to determine the degree of conservation between different species. Sequencing alignment analysis of exon 5 revealed a high level of conservation between the human *HMOX1* gene and that of *Pan troglodytes* (100%), *Pongo pygmaeus* (98%), *Macaca mulatta* (94%) and *Canis familiaris* (82%). The glycine substituted by this variant is conserved across species, which could be an indication that this amino acid could have functional significance at this position. ESEfinder results, however, predicted that this variant has no effect on the binding of SR-proteins and subsequently, it is not believed to have an effect on splicing (Cartegni et al. 2003). The change in polarity caused by the amino acid substitution, may have an effect on protein folding. This variant was identified in a single male OC patient with serum ferritin levels of 213.1 μg/l and it is possible that this variant is responsible for the high ferritin levels observed.

Another novel exonic variant identified in exon 5 of the *HMOX1* gene, is R262C. This variant causes a change from arginine, a polar amino acid, to cysteine, a hydrophobic amino acid. The homology search indicated that the arginine replaced by this variant, is not conserved across species. *P. pygmaeus* contains a histidine and *C. familiaris* a proline at this amino acid position, with *M. mulatta* being the only species retaining the arginine. ESEfinder results predicted that the presence of the R262C variant abolishes a SF2/ASF motif as well as an SRp40 binding motif, an indication that this variant may have an effect on the structure and/or function of the protein. The variant was identified in a female OC patient who demonstrated raised serum ferritin levels [278.4 μg/l (reference range 20–200 μg/l)]. It may be possible that this variant is responsible for the high serum ferritin levels observed.

R262H identified in exon 5 of the *HMOX1* gene, causes a change from the polar amino acid arginine, to another polar amino acid, histidine. Results obtained using the ESEfinder program indicates that this variant abolishes a SF2/ASF motif, a SC35 motif, as well as a SRp40 motif, while creating a new SRp40 motif. This variant was identified in a single female OC patient with raised serum ferritin levels [1494.4 μg/l (reference range 20–200 μg/l)], and was also heterozygous for the variants IVS2+4T→C, IVS1-24G→C, I109, 3'UTR+284C→T and L17. It may be possible that the combined presence of these variants contributed to the excessive iron overload of this patient.

The Q248H polymorphism in exon 6 of the *SLC40A1* gene was discovered in African-Americans and Africans with iron overload and subsequent studies have revealed that this polymorphism lies in the predicted cytoplasmic domain and is conserved across mammals (Gordeuk et al. 2003). This variant substitutes an uncharged glycine with a positively charged histidine. Beutler et al. (2003) found that the Q248H showed a higher frequency in patients with high ferritin levels and homozygous Q248H individuals had significantly higher serum ferritin levels than heterozygous and wild-type homozygous individuals. This was not the case in the present study, where only one individual demonstrated iron overload [serum ferritin 247.2 μg/l (reference range: females 20-200 μg/l), one had iron deficiency (serum ferritin < 20 μg/l) and the other six OC patients had normal serum ferritin levels. There is also a tendency of the heterozygous variant to be overrepresented among subjects with elevated serum ferritin levels. Gordeuk et al. (2003) suggested that this variant might be associated with a tendency to anaemia. Q248H is expressed on the cell surface as efficiently as the wild-type ferroportin and allows the full export function of the protein (Schimanski et al. 2005). The Q248H variant is thus considered a polymorphism of *SLC40A1*, and is infrequently associated with clinical disease. This variant was not detected in any of the

Black South African iron overload patients in the study of Zaahl et al. (2004), but was observed in the Yoruba population of the HapMap project (allele frequency 0.042; 2n = 120). A higher allele frequency of this variant was observed in the OC population screened in this study, although a smaller population size was studied. It may also be possible that Q248H is in linkage disequilibrium with other disease-causing loci, therefore contributing to iron overload.

A310G was identified in exon 4 of the *HJV* gene in both the patient and control groups. This variant substitutes an alanine with glycine (Lee et al. 2004). As both are non-polar hydrophobic amino acids, this variant is not expected to have a structural effect on the protein. Recent studies have detected this variant in African-Americans presenting with iron overload as well as in the population-matched control individuals (allele frequencies 0.0196 and 0.0720, respectively). These studies have observed that mutations in the coding regions of the *HJV* gene are uncommon in African-Americans (Barton et al. 2004, Lee et al. 2004), similar to the low frequency of *HJV* mutations observed in the Black South African OC population in this study. The allele frequency in our population is similar to that observed for African-Americans (0.04; 2n = 46) using HapMap, but differed from the Sub-Saharan population (0.09; 2n = 120). It appears that this variant has thus far only been detected in individuals of African descent. A310G was identified in two female and two male OC patients in the present study, with only one of these individuals demonstrating iron overload [serum ferritin = 589.5 (reference range 20–300 µg/l)]. The combined presence of this variant with other variants identified in this study, may be responsible for the iron overload observed in this patient. The other three patients had normal serum ferritin levels of 109.6 µg/l, 171.8 µg/l and 174.6 µg/l (reference range: females 20–200 µg/l; males 20–300 µg/l), for the two females and one male, respectively.

Six of the variants detected in this study, do not cause an amino acid change and in general, are therefore not expected to affect the functions of the relevant genes. These variants include the novel L378 in *SLC40A1*, as well as L17 and P195 in *CYBRD1*. The three previously described synonymous variants identified include I109 (Beutler and West 2003), L129 (Zaahl et al. 2004) and V221 (Devalia et al. 2002) in *SLC40A1*. Synonymous single-nucleotide polymorphisms located in coding regions are generally regarded as translationally silent as the relevant amino acids remain the same, but these variants have the ability to affect alternative splicing or could be involved in a number of processes affecting translation. This includes the possible creation of new splice sites, the disruption of exonic splicing enhancers and silencers (Caceres and Kornblihtt 2002), and mRNA levels, thereby affecting the translated product (Cartegni et al. 2002). A silent mutation was shown to induce post-transcriptional exon-skipping in the case of phenylketonuria (PKU) where the c.1197A→T substitution resulted in the skipping of exon 11, leading to a PKU phenotype (Chao et al. 2001).

Each of the novel synonymous variants in this study was analysed using ESEfinder, to determine whether it may have an effect on splicing. The results generated, indicated that the L378 variant in exon 7 of *SLC40A1*, causes the creation of an extra P40 binding motif. This variant was detected in a single OC patient who was also heterozygous for three other variants (5'UTR-98G→C, IVS1-24G→C and Q248H) identified in the *SLC40A1* gene. This patient did not have irregular iron parameters, indicating that this variant possibly has no effect on iron overload or deficiency.

The synonymous variant L17 was identified in exon 1 of the *CYBRD1* gene. Results obtained using the ESEfinder program, indicate that this variant is predicted to create a new binding motif for each of the SR-proteins SF2/ASF and SRp40, while it also abolishes a binding motif

for SRp40. This variant was identified in the heterozygous state in two OC patients, one male and one female, with intermediate (160.7 μg/l) and extremely raised [1494.4 μg/l (reference range 20–200 μg/l)] serum ferritin levels, respectively. This variant also appears to have no significant effect on iron regulation, but it is possible that the combined presence of L17 with other variants identified may be responsible for the difference in the iron parameters of the OC patients.

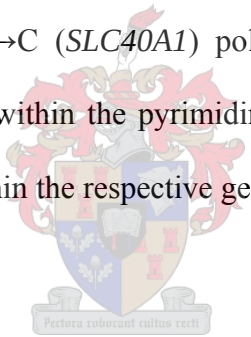
ESEfinder results indicated that the synonymous variant P195 in exon 4 of the *CYBRD1* gene is predicted to create a new SF2/ASF motif and two new SRp40 motifs, while also being responsible for the abolishment of an SC35 motif. This variant was identified in three OC patients with varying serum ferritin levels [two females: 95.7 μg/l and 247.2 μg/l; one male: 260.1 μg/l (reference range: females 20–200 μg/l; males 20–300 μg/l)]. The combined presence of P195 with other variants identified in this study may be responsible for the difference in the iron parameters of the OC patients.

None of the previously described synonymous variants have been implicated in having an effect on the function of the SLC40A1 protein. A previous study involving iron overload in the Black South African population, identified I109 (allele frequencies: patients 0.0; controls 0.003), L129 (allele frequencies: patients 0.23, controls 0.05) and V221 (allele frequencies: patients 0.14, controls 0.0) and revealed significant associations for L129 and V221 (Zaahl et al. 2004). Higher allele frequencies were observed for these three variants in the Black South African OC population in the present study, although no significant associations were observed. The higher allele frequencies may be due to the larger population size of this study (2n = 100) in comparison to the previous study (2n = 22).

Intronic variants

Eight previously described (***HFE***: IVS4-44T→C, IVS5-47A→G; ***HMOX1***: IVS2-19T→C, IVS4+51delTGGCTGTCTGACT, IVS4+59C→G; ***SLC40A1***: IVS1-24G→C and ***CYBRD1***: IVS1-4C→G, IVS2+8T→C) and one novel (***SLC40A1***: IVS5-27A→C) intronic variant were detected in this study.

Accurate splicing in eukaryotes is dependent on the 5' splice site, the 3' splice site, and the branch site (Cartegni et al. 2002). Motifs in the intron acting as a branch site have been identified with a conserved A residue in the branch site serving as a branch point. The IVS2-19T→C (*HMOX1*) and IVS1-24G→C (*SLC40A1*) polymorphisms lie within this region, while IVS1-4C→G (*CYBRD1*) lies within the pyrimidine tract and therefore, these variants could all possibly affect splicing within the respective genes. Functional studies are needed to elucidate the roles of these variants.



The intronic variants identified in this study were all subjected to the Alternative Splice Site Predictor (ASSP) analysis program (http://es.embnet.org/mwang/assp.html) to determine whether or not they could affect the splicing mechanism (Wang and Marín 2006). The variants not predicted to affect splicing include: ***HFE***: IVS4-44T→C (Beutler and West 1997), IVS5-47A→G (Beutler and West 1997); ***HMOX1***: IVS2-19T→C (SeattleSNPs), IVS4+59C→G (SeattleSNPs); ***SLC40A1***: IVS1-24G→C (Devalia et al. 2002), IVS5-27A→C and ***CYBRD1***: IVS1-4C→G (Zaahl et al. 2004), IVS2+8T→C (Zaahl et al. 2004). A 13 base pair deletion from position 51 (IVS4+51delTGGCTGTCTGACT) was identified in intron four of the *HMOX1* gene. This deletion was found at the splice acceptor site of the intron. The functional significance of this variant is not yet known and no statistically significant

associations were obtained in this study. Variants exerting an effect on the splicing process are not only those located at splice sites, but also include those that may affect regulatory elements within exons or introns, such as 'enhancers' and 'silencers' (Pagani and Baralle 2004).

The following variants were all identified in the Black South African population in the study of Zaahl et al. (2004): ***HFE***: IVS2+4T→C, IVS4-44T→C, IVS5-47G→A; ***SLC40A1***: IVS1-24G→C and ***CYBRD1***: IVS1-4C→G, IVS2+4T→C. The allele frequencies of the present study are higher than those observed for the iron overload study: IVS2+4T→C (0.18), IVS5-47G→A (0.15), IVS1-4C→G (0.0), IVS2+8T→C (0.77). It is possible that following further studies, these variants may be implicated in OC susceptibility as indicated by the increased frequency of these variants in the OC population or this variation may be attributed to the difference in population sizes of the two studies. The frequencies of the IVS4-44T→C (0.18) and IVS1-24G→C (0.82) variants of the iron overload study, were higher than those observed in this study. This may be an indication of the involvement of these variants in iron overload.

3' UTR variants

Four novel (***SLC40A1***: 3'UTR+284C→T, 3'UTR+289G→A, 3'UTR+289G→T and ***HJV***: 3'UTR+47 A→G) variants were identified in the 3'UTR in this study. Variations in the 3'UTR of genes can alter the stem-loop structure of mRNA and can affect mRNA processing and stability (Dean et al. 2001). The 3'UTR contains a diversity of regulatory mechanisms, including the polyadenylation signal, responsible for the regulation of mRNA stability (Mazumder et al. 2003). Other regulatory signals that have also been detected in the 3'UTR are involved in the subcellular localisation of the transcripts. Functional studies need to be

performed to investigate if 3'UTR+284C→T, 3'UTR+289G→A, 3'UTR+289G→T and 3'UTR+47 A→G have an effect on any of these regulatory elements and to determine the mechanisms by which altered mRNA may influence risk of developing cancer. 3'UTR+284C→T was identified in two female and one male OC patient with serum ferritin levels of 124.3 µg/l, 1494.4 µg/l and 278.4 µg/l, respectively. 3'UTR+289G→A was identified in two male OC patients with serum ferritin levels of 260.1 µg/l and 589.5 µg/l and 3'UTR+289G→T was identified in three male OC patients with serum ferritin levels of 151.3 µg/l, 212.6 µg/l and 402.2 µg/l. The variants identified in the 3'UTR may be responsible for these ferritin levels. The 3'UTR+47A→G variant was identified in five OC patients, one female and four males. These patients had varying serum ferritin levels, with the female patient demonstrating iron deficiency (serum ferritin < 20 µg/l), three males had normal serum ferritin levels and one male had iron overload [serum ferritin = 342.6 µg/l (reference range: males 20–300 µg/l)]. It is possible that these variants may be in linkage disequilibrium with other disease-causing variants, subsequently contributing to OC susceptibility.

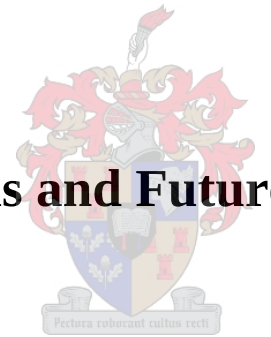
Three techniques (HEX-SSCP analysis, restriction enzyme digestion and semi-automated DNA sequencing) were employed for mutation analysis in this study. The advantages of employing the HEX-SSCP technique, is that it is inexpensive and easy to use. However, it provides only 70% sensitivity (Hayashi and Yandell 1993). This was illustrated when screening exon 2 of *HFE* and exon 6 of *SLC40A1*, where restriction enzyme digestion had to be employed to distinguish between homo- and heterozygous individuals for IVS2+4T→C and Q248H. All the variants were verified using bi-directional sequencing analysis. No variants were identified in the *HAMP* gene, following HEX-SSCP analysis. It is possible that this technique was not sensitive enough to detect variants in this gene or the variant may not have been present in the individuals screened in this study.

There was an equal distribution of males and females in the patient cohort, however in the population-matched control group the distribution was unequal. The results obtained in this study thus cannot give an accurate assessment of the prevalence of the variants in males compared to females. Iron parameters between the individuals of the patient group, as well as between individuals of the control group, also varied greatly. Some of the individuals had iron overload, some had intermediate ferritin levels, while others were anaemic. An unequal representation of each of these groups complicated the determination of statistical association of the variants with the iron parameters.

The population sizes were sufficient for the purpose of this pilot study. It allowed the detection of previously described as well as novel variants. Although no statistically significant associations have been observed for any of the variants identified in this study, the allele frequencies obtained for the variants in the Black South African population, could be employed to compare to other studies investigating this population. The detection methods employed could have an effect on the results obtained in this study. Larger sample sizes, however, can aid in obtaining more informative statistical data.

Although no statistically significant associations were obtained for the novel variants in this study, the possible effects of these variants should not be disregarded. Statistically significant associations have also been observed between the IVS5-47G→A variant in *HFE* and (a) V221 (allele frequencies: $P = 0.046$) and (b) Q248H (allele frequencies: $P = 0.031$) in *SLC40A1*. It is possible that the combined presence of these variants is involved in disease susceptibility. Results obtained with ESEfinder also indicated that R262C, R262H, L378, L17 and P195 might have an effect on splicing and thus the possible role these variants may play in OC susceptibility should not be disregarded.

Chapter 4



Conclusions and Future Prospects

Oesophageal cancer is a complex disease, developing through a combination of genetic and environmental factors. The symptoms of OC appear at an advanced developmental stage of the disease, subsequently making treatment highly ineffective. Early detection is the only effective method of intervention, making the establishment of an early diagnostic screening programme imperative. Previous studies have investigated the role of various disease risk factors in the development of OC, including smoking and drinking, oesophageal injury and excess iron. This study attempted to investigate the role of iron dysregulation in six genes involved in iron metabolism, in the Black South African OC population, hopefully setting the foundation for a future diagnostic programme.

The first objective of this study was to investigate the entire coding regions of the *HFE*, *HMOX1*, *SLC40A1*, *HAMP*, *CYBRD1* and *HJV* genes to identify any previously described and novel mutations and/or polymorphisms that could be involved in OC susceptibility. This was achieved by PCR amplification of the coding and flanking intronic regions of the six genes and subsequently, HEX-SSCP analysis, RFLP analysis and semi-automated DNA sequencing.

The results obtained are a clear indication that the first objective of this study was achieved, as a total of 34 variants were identified. This included 21 previously described variants (***HFE***: V53M, H63D, H63, IVS2+4T→C, IVS4-44T→C, C282Y, IVS5-47G→A; ***HMOX1***: IVS2-19C→T, IVS4+51delTGGCTGTCTGACT, IVS4+59C→G; ***SLC40A1***: 5'UTR-98G→C, 5'UTR-23A→G, 5'UTR-8C→G, IVS1-24G→C, I109, L129, V221, Q248H; ***CYBRD1***: IVS1-4C→G, IVS2+4T→C and ***HJV***: A310G) and 13 novel variants (***HFE***: Y342; ***HMOX1***: G255R, R262C, R262H; ***SLC40A1***: IVS5-27A→C, L378, 3'UTR+284C→T, 3'UTR+289G→A, 3'UTR+289G→T; ***CYBRD1***: L17, P195 and ***HJV***: 5'UTR-1401T→C, 3'UTR+47A→G). The variants occurring in DNA sequences can have an

impact on an individual's response to disease, viral agents, drugs and therapy. Each population represented in South Africa is unique and this makes it important that individuals from different South African populations should be screened for the variants identified in these genes. This data could be of immense value to clinicians and pharmacists, as it could be used in the elucidation of differential response to drugs and therapy among different individuals.

Although a large number of variants were identified in this study it is however, not the full spectrum of mutations in the Black South African population. Various polymorphisms or disease-causing mutations previously described in this population have not been identified in this study. This occurrence may be attributed to a number of reasons including the following: 1) these variants were not present in any of the individuals screened in this study, 2) these variants possibly do not contribute to OC susceptibility and subsequently are not generally present in OC patients or 3) these variants could not be detected with the mutation detection methods employed in this study.

The study cohort included 50 OC patients and 50 population-matched control individuals. Although this population size is indicative of a pilot study, it sufficiently allowed the identification of the 34 aforementioned variants. It also allowed the calculation of the allele frequencies of these variants in the Black South African population. A larger patient cohort may be integrated into this study to determine if the variants identified, especially the novel variants, occur at increased frequencies in the Black South African OC population. This may indicate the significance of these variants or indicate the individual or combined contribution to OC susceptibility. Many of these variants have been detected in Africans and/or African-Americans regardless of the disease. Although many polymorphic markers are common in

both OC patients and in the control population, the calculated allele frequencies give an indication of the incidence of these variants in the Black South African population. This data can be compared to results obtained from other studies including South African populations and various diseases, giving an indication of the potential significance of these variants. A larger study population may also allow the detection of other previously described polymorphisms and disease-causing mutations that were not identified in this study.

HEX-SSCP analysis, RFLP analysis and semi-automated DNA sequencing were the detection methods of choice for this study. The first objective of this study was sufficiently achieved as these techniques allowed the identification of various novel and known variants. Previous studies have shown that the SSCP technique is only 70% sensitive and that the sensitivity of SSCP analysis decreases with the increase of fragment sizes over 300 bp (Hayashi and Yandell 1993). This may be an indication that the fragments screened in this study could possibly have been too large in some instances where sizes ranged between 250 and 350 bp, influencing the accuracy of the results obtained. The majority of fragments screened were larger than 250 bp, with only the amplified products of *HFE* exon 6, *HMOX1* exons 2 and 3A, *SLC40A1* exon 4, *HAMP* exon 2, *CYBRD1* exon 4B and *HJV* exon 4D being smaller than 250 bp. The PCR fragment obtained for exon 6 of the *SLC40A1* gene was 368 bp long. This may be the reason why RFLP analysis had to be employed to distinguish between the homozygous and heterozygous states of the Q248H variant. The position of a variant within a fragment can also influence the sensitivity of the method. As the objective of this pilot study was mainly to identify novel and/or described variants, it was not deemed essential to optimise the HEX-SSCP technique specifically for each variant. This could be done in future studies, to allow large-scale population screening.

Research could be conducted into other screening techniques that are possibly more efficient at detecting variants, yet are still cost effective. Semi-automated DNA sequencing has the ability to identify both novel and known changes in the DNA sequence and is considered the most accurate mutation detection technique developed (Kristensen et al. 2001). The advantages include the ability to detect all variants present in a specified sequence. The disadvantages are that it is quite expensive and not as applicable in routine diagnostic laboratories. Denaturing high performance liquid chromatography (dHPLC) is also considered the superior technique for the detection of SNPs with regard to its high sensitivity, efficiency and cost-effectiveness (Underhill et al. 1997). The advantages of dHPLC include a sensitivity level of 92-100% and fragment sizes ranging from 198-732 bp can be screened. This is an indication of the superiority of dHPLC over the SSCP technique, which has a sensitivity level ranging between 70-100 % for fragment sizes of 130-250 bp (Xiao and Oefner 1992, Bonner and Ballard 1999). With the enlargement of the OC patient group, RFLP analysis can be employed to screen for all the previously described variants already identified in this population group as this technique is much less time consuming than SSCP analysis. DNA chip technology may also be considered for future studies. A DNA chip may be designed for the most common mutations, allowing the simultaneous detection of different alleles from various genes that have been implicated in OC susceptibility. This technology is still not as freely available and is still quite expensive. Kotze et al. (2004) have developed a rapid reverse hybridisation method enabling the simultaneous analysis of multiple mutations (a total of 18) related to hereditary iron overload. Ready-to-use membrane-test strips are employed to obtain a result from a blood samples within six hours. Mutations identified in the *HFE*, *TFR2*, and *SLC40A1* genes are all included in this commercially available test kit, which can employ inexpensive equipment such as a waterbath and shaker for the hybridisation steps.

The second objective of this study was to identify significant associations between the variants identified and OC susceptibility. This was achieved by determining if all variants were in Hardy-Weinberg equilibrium in the respective populations and subsequently, comparing the allele frequencies of the OC patient group with the population-matched control individuals. Additionally, this study attempted to identify the existence of gene-gene interaction according to Butt et al. (2003). Although no statistically significant associations were obtained for the individual variants, statistically significant associations were observed between the IVS5-47G→A variant in the *HFE* gene and (a) V221 (allele frequencies: $P = 0.046$) and (b) Q248H (allele frequencies: $P = 0.031$) in the *SLC40A1* gene. This data gives an indication that the combined presence of these variants may be involved in disease susceptibility. Further gene-gene analysis in a larger study cohort, may reveal the interaction of these genes in this disease. Future studies may possibly indicate the role of certain variant combinations in conferring OC susceptibility or the effect of these combinations on iron regulatory pathways.

The results obtained with the ESEfinder program indicated that the majority of novel variants identified in this study (***HMOX1***: R262C, R262H; ***SLC40A1***: L378 and ***CYBRD1***: L17, P195) might have an effect on splicing and thus the possible role these variants may play in OC susceptibility should not be disregarded. Functional studies should be performed to elucidate the possible effects of the novel variants detected in the OC population of this study, which include: one variant in the 5'UTR (**HJV**: 5'UTR-1401T→C), six exonic variants (***HMOX1***: G255R, R262C, R262H; *SLC40A1*: L378 and ***CYBRD1***: L17, P195), one intronic variant (***SLC40A1***: IVS5-27A→C) and four variants in the 3'UTR (***SLC40A1***: 3'UTR+284C→T, 3'UTR+289G→A, 3'UTR+289G→T and ***HJV***: 3'UTR+47A→G).

Numerous studies have highlighted the effects of silent mutations on the protein function. The study of Chao et al. (2001) illustrated how a silent mutation induced post-transcriptional exon-skipping leading to a phenylketonuria (PKU). Another example is the silent mutation C6354, in the Fibrillin-1 (*FBN*-1) gene, in patients with Marfan syndrome (MFS). Although it was not considered to affect known binding sites, the results of different types of expression constructs, confirmed that the skipping of exon 51 is solely due to the silent mutation (Liu et al. 1997). The three novel synonymous variants (***SLC40A1***: L378 and ***CYBRD1***: L17, P195) identified in this study, are all predicted to have an effect on splicing. Future studies can employ the minigene system to discern the disease-causing potential of the variants predicted to cause abnormal splicing. The minigene system is considered an indispensable tool for *in vivo* analysis of regulatory elements that allow efficient splicing and are involved in the regulation of alternative splicing (Cooper 2005).

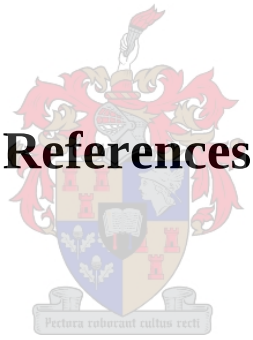
To further investigate the role of iron dysregulation in the development of OC in the genetically distinct South African populations, our study population can be extended to include the White and Coloured populations. Two previous studies including the Coloured (Zaahl et al 2003) and the Black (du Plessis 2000) populations have already confirmed the association of *NRAMP1* variants with OC susceptibility. Studying the occurrence of these variants in other OC populations could provide us with valuable statistical information and could hopefully ultimately lead to improving the diagnostic and counseling service that is offered to the populations of this country.

Future research will also include screening the existing population for novel and/or previously described variants in other genes involved in iron metabolism, including the hephaestin (*HEPH)*, ceruloplasmin (*CP*) and transferrin receptor 2 (*TFR2*) genes. Larger sample sizes of

the Black South African population will be included, which will also be screened for all of the variants identified in the present study. The promoter regions of the three new genes (*HEPH*, *CP* and *TRF2*) as well as the six genes already studied (*HFE*, *HMOX1*, *SLC40A1*, *HAMP*, *CYBRD1* and *HJV*) will also be investigated. Previous studies of the *HMOX1* gene promoter region have already implicated mutations and a promoter repeat $(GT)_n$ in various diseases including neurodegenerative (Kimpara et al. 1997) and pulmonary diseases (Yamada et al. 2000).

This study successfully identified novel and previously described variants in the Black South African OC population. Significant associations were observed that might possibly implicate the combined presence of certain variants in OC susceptibility. Future research may indicate the significance of the individual variants identified in this study and may also illustrate their role in conferring OC susceptibility. Following further research, this study may prove to be an important contribution to the current knowledge of iron dysregulation in the development of OC.

Chapter 5



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5.2 Electronic-Database Information

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