A STUDY OF HOST GENETIC DETERMINANTS OF HUMAN PAPILLOMAVIRUS (HPV) INFECTION, CERVICAL CANCER AND HERPES SIMPLEX VIRUS TYPE-2 (HSV-2) INFECTION

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This thesis is dedicated to my beloved parents, MAA and BAPI.

“YOU ARE THE CREATOR OF YOUR OWN DESTINY”

Swami Vivekananda

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ABBREVIATIONS

Abs	Antibodies
AICD	Activation Induced Cell Death
AIDS	Acute Immunodeficiency Disease Syndrome
APCs	Antigen Presenting Cells
ASCUS	Atypical Squamous Cells of Undetermined Significance
ASIR	Age Standardised Incidence Rate
ASP	Affected Sib-pair
CCR	Chemokine Receptor gene
CIN	Cervical Intraepithelial Neoplasia
CM	Cytomembrane
CMI	Cell-mediated Immunity
CTL	Cytotoxic T-lymphocyte
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked Immunosorbent Assay
Fas-L	Fas-Ligand
GAS	Gamma Interferon Activation Signal
GCC	Gastric Carcinoma Cell
GEC	Gastric Epithelial Cell
GUD	Genital Ulcer Disease
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus

HR-HPV	High-risk Human Papillomavirus (HR-HPV)
HSIL	High-grade Squamous Intraepithelial Lesion
HSPGs	Heparan Sulphate Proteoglycans
HWE	Hardy-Weinberg Equillibrium
IARC	International Agency for Research on Cancer
ICC	Invasive Cervical Cancer
IFn	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon Regulatory Factor
KIR	Killer Cell Immunoglobulin-like Receptor
LCR	Long Control Region
LD	Linkage Disequilibrium
LHc	Langerhans Cells
LOD	Logarithm Of the Odds
LSIL	Low-grade Squamous Intraepithelial Lesion
MCP-1	Monocyte Chemoattractant Protein-1
MHC	Major Histocompatibility Complex
MICA	MHC Class I Polypeptide-Related Sequence A
MTHFR	Methylenetetrahydrofolate Reductase
NGIL	Normal Gastric Stroma-Infiltrating Lymphoid Cell
NK	Natural Killer
OR	Odds ratio
PCR	Polymerase Chain Reaction
SIL	Squamous Intraepithelial Lesion

SNP	Single Nucleotide Polymorphism
SP-1	Stimulatory Protein 1
STAT-1	Signal Transducer and Activator of Transcription 1
STD	Sexually Transmitted Disease
TAMs	Tumor-associated Macrophages
TH	T Helper cell
TIL	Tumour-Infiltrating Lymphoid Cell
TILs	Tumour-infiltrating Lymphocytes
TNF	Tumour Necrosis factor
Tp53	Tumour Supressor Protein 53
WHO	World Health Organisation

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CHAPTER 5

A STUDY OF HOST GENETIC DETERMINANTS OF HUMAN PAPILLOMAVIRUS (HPV) INFECTION, CERVICAL CANCER AND HERPES SIMPLEX VIRUS TYPE-2 (HSV-2) INFECTION

Koushik Chatterjee

ABSTRACT

Background: Cervical cancer, caused by oncogenic types of human papillomavirus (HPV), is the second most common cancer in women worldwide. Although large numbers of young sexually active women get HPV-infected only a small fraction develop cervical cancer pointing to co-factors including host genetics playing a role in the outcome of the disease. The aim of this thesis was to investigate the role of genetic polymorphisms in the cell death pathway genes, Fas (FasR-1377G/A and FasR-670A/G), FasL (FasL-844T/C) and CASP8 (*CASP8* -652 6N ins/del) in cervical cancer, pre-cancers and HPV infection. The genes have also been investigated in herpes simplex virus type-2 (HSV-2) infection, a co-factor for developing cervical cancer. Another immune response gene, CCR2 (*CCR2V64I*) was also investigated.

Materials: DNA from 1879 subjects comprising 447 women with cervical cancer (106 black African and 341 women of mixed-ancestry) and 1432 controls (306 black African and 1075 mixed-ancestry) matched in a 1:3 ratio to the cases on age, ethnicity and domicile status (urban/rural) was available. 265 HSV-2 infected and 142 HSV-2 non-infected controls were used to study the genotype-HSV-2

association. The ethical approval was obtained for this study from the University of Cape Town Research Ethics Committe (REC REF: 075/2009).

Methods: The genomic DNA was extracted using TotalNucleicAcid Extraction kit and quantified using Nanodrop Spectrophotometer. Fas and FasL genotyping was carried out for 447 cases and 424 controls using TaqMan assaymix. 445 cases and 1221 controls were genotyped for CASP8 using PCR-RFLP method. For CCR2 genotyping PCR-SSP (sequence specific primers) method was using for 446 cases and 1432 controls by PCR-RFLP and PCR. Statistical analysis was done for the alleles, genotypes and haplotypes adjusted for ethnicity and smoking using R, haplo.stats, Stata 9 and Haploview 4.2 software.

Results: The distribution of Fas and FasL polymorphisms were not different between cervical cancer patients and controls but were significantly different among black and mixed-ancestry women. However, the FasR-1377A allele was significantly associated with reduced risk of HSV-2 infection (OR (95% CI) = 0.58 (0.38-0.87), P = 0.008) . *CASP8* -652 6N del/del genotype showed a significant association with increased risk of abnormal cytology (OR (95%CI) = 1.66 (1.00-2.75), P = 0.048) and with increased risk of high-risk HPV infection (OR (95%CI) = 2.84 (1.11-7.28), P = 0.030) (only in black Africans) but no association with cervical cancer. The *CCR2-64I* variant which is involved with the homing of macrophages was associated with increased risk of cervical cancer (OR (95%CI) = 6.14 (4.79-7.86), P = 0.001) and this significant susceptible effect was also present when squamous intraepithelial lesion (SIL) positive controls were compared to cervical cancer patients (OR (95%CI) = 6.86 (4.19-11.21), P = 0.001).

Conclusion: This is the first study of the role of Fas, FasL, CASP8 and CCR2 polymorphisms in cervical cancer in an African population. This is also the first

study reporting a non-HLA host genetic link to HSV-2 infection. Our results show that genetic polymorphisms in genes involved in the activation-induced cell death (AICD) are associated with differential risk to cervical cancer, pre-cancerous lesions, HPV infection and HSV-2 infection. We report that genetic variability in genes involved in macrophage recruitment is also associated with pre-cancerous lesions and cervical cancer.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Cervical cancer

1.1.1 Cervical Cancer burden in the world

Cervical cancer is the second most frequent cancer and is a major cause of cancer related deaths in women worldwide. Approximately 493,243 new cervical cancer cases and 273,505 cervical cancer deaths were reported in 2007 (Castellsague X et al., 2007). The incidence of cervical cancer in most of Europe, North America, Australia and New Zealand is low at the present time due to effective screening programmes (Fig. 1.1) (Ferlay J et al., 2004)

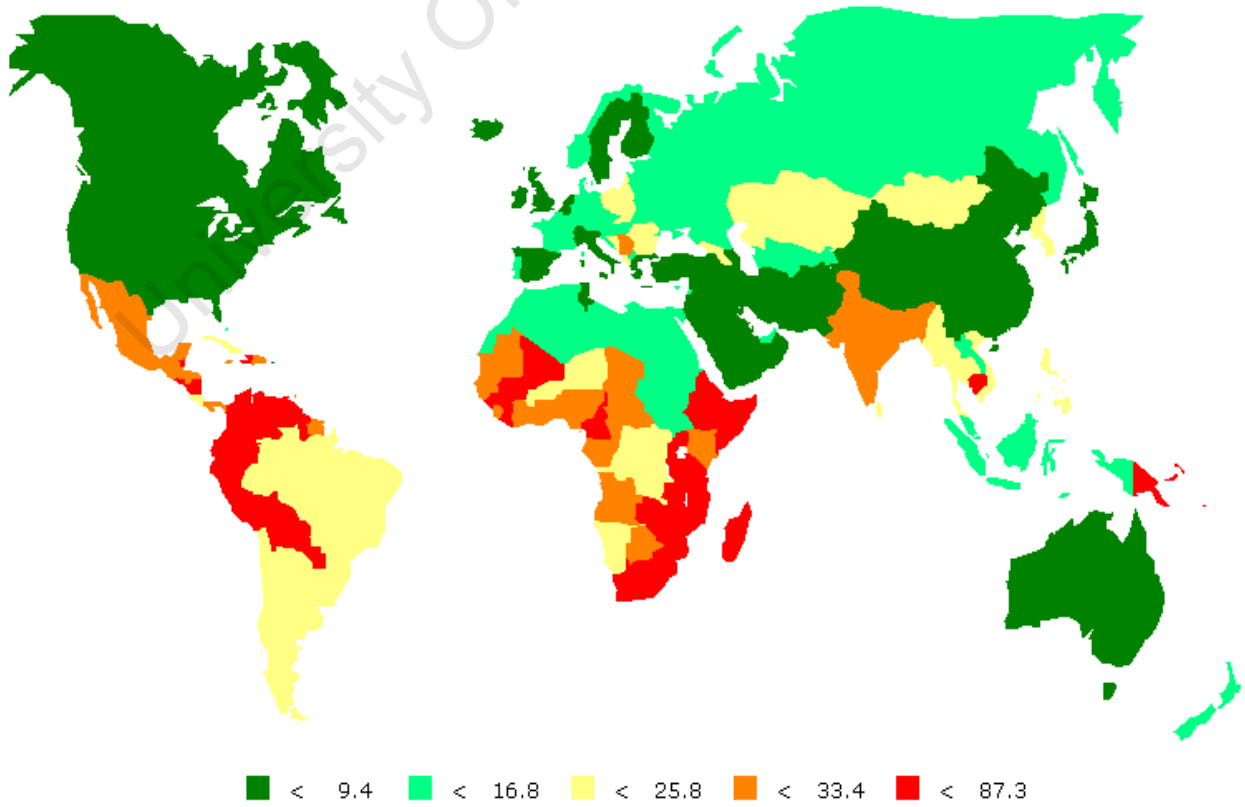

Fig. 1.1: Worlwide age-standardised incidence (per 100,000) of cervical cancer (Ferlay J et al., 2004)

Before the introduction of screening programmes the incidence in these regions was much higher and similar to the high incidence rates of present day Africa. For example, at the time of the Second US National Cancer Survey in 1947 the age standardised incidence rate (ASIR) in the US was 38.0 in 10^5 females (Dorn and CUTLER, 1959) which is much lower than the current ASIR of 15 per 100,000 in developed countries (Parkin and Bray, 2006). Effective screening programs have significantly reduced the incidence and mortality rates in developed countries. The lowest rates (ASIR = less than 15 per 100,000) are found in Europe, North America and Japan (Parkin and Bray, 2006).

1.1.2 Cervical Cancer burden in southern Africa

Due to poor screening programs and lack of treatment, 83% of all new cervical cancer cases and 85% of all cervical cancer-related deaths now occur in developing countries (Anorlu, 2008). Cervical cancer is the most common cancer with the highest cause of cancer mortality in women in sub-Saharan Africa. An estimated number of 70,700 new cases of invasive cervical cancer (ICC) occur each year which accounts for 25.4% of all cancers in women in sub-Saharan Africa (Parkin et al., 2008). ICC incidence in sub-Saharan Africa is one of the highest in the world. The ASIR is estimated as 31 per 100,000 of sub-Saharan African women and varies by region with Southern Africa being 38.2 per 100,000 (Fig. 1.2) (Ferlay J et al., 2004;Parkin and Bray, 2006).

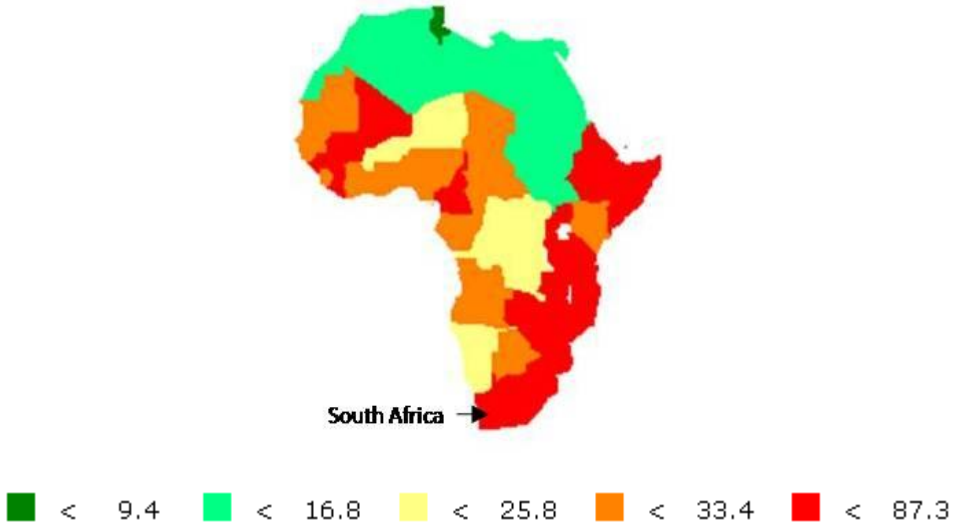

Fig. 1.2: Age-standardised incidence rates of cervical cancer in Africa (Ferlay J et al., 2004).

Interestingly, the incidence rates remain unchanged in between 1960-1990 in South Africa (Parkin et al., 2008). It is projected that there will be 118,000 new ICC cases in 2025 in sub-Saharan Africa which is a 67% increase in the burden of cervical cancer compared to 2002 (Ferlay J et al., 2004).

1.1.3 Classification and Stages of Cervical Cancer

There are two main types of cervical cancer, squamous cell carcinoma and adenocarcinoma. Squamous cell carcinoma derives from squamous cells representing the majority of the cells in lower cervix and is the most common form of cervical cancer acquiring for 75% of all the cervical cancers. Adenocarcinoma arises from cervical glandular cells and is rarer (Vizcaino et al., 1998). Cervical cancer is a multistep process that develops progressively through different stages from normal epithelium to invasive cervical carcinoma following an HPV infection of oncogenic types (Paavonen, 2007). HPV infection may lead to atypical squamous cells of undetermined significance (ASCUS). Persistent infection by oncogenic types of

HPV in ASCUS leads to squamous intraepithelial lesions (SIL) (Rohan et al., 2003). SIL is further divided into low-grade (LSIL) and high-grade (HSIL) stages. LSIL are precancerous and are considered as very early precursor stages of cervical cancer that rarely progress to cancer (Snijders et al., 2006) while most HSILs progress to ICC when left untreated (Kobayashi et al., 2004). When HSIL penetrates the basement membrane and spreads to the other sites of the body it is called invasive cervical carcinoma (ICC) (Fig. 1.3).

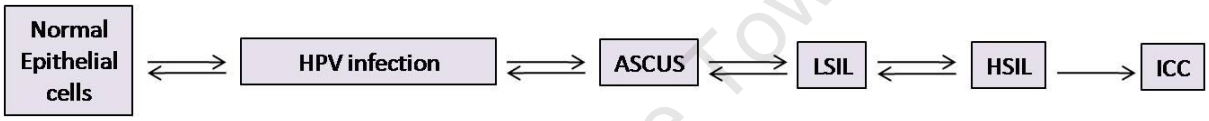

Fig. 1.3: A schematic diagram showing the progression to ICC from normal epithelial cells by HPV infection.

There is strong epidemiological and experimental data that have demonstrated a definite association of high-risk human papillomavirus (HR-HPV) infection and the development of cervical cancer (Schiffman et al., 1993;zur Hausen, 2002). HPVs are associated with > 99% of all cervical cancer cases worldwide (Fig. 1.5) (HPV Information Centre, 2009). The association of HPV with other cancers is much lower compared to the association with cervical cancer (Fig. 1.4) (HPV Information Centre, 2009).

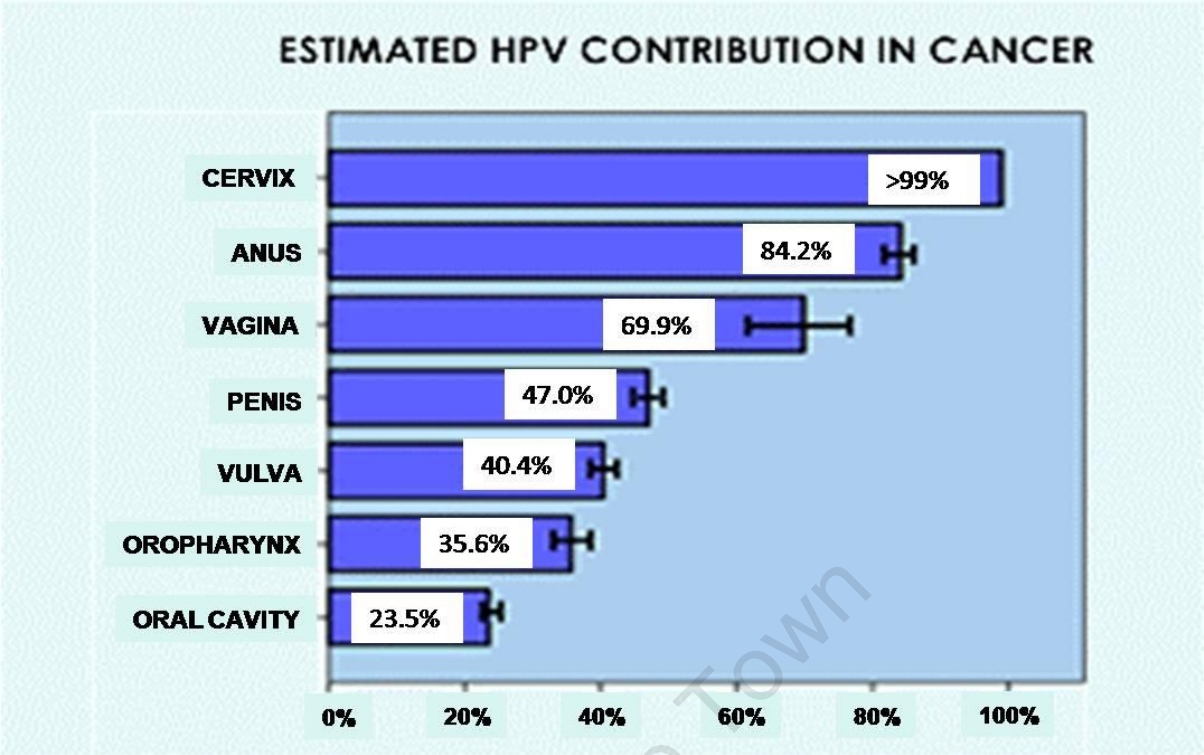

Fig. 1.4: A schematic diagram showing the association of HPV with different types of cancers (HPV Information Centre, 2009)

Two prophylactic HPV vaccines are currently available in the market, the bivalent vaccine for HPV 16 and 18 (Cervarix, GlaxoSmithKline Biologicals, Rixensart, Belgium) and the quadrivalent vaccine for HPV 6, 11, 16, and 18 (Gardasil, Merck and Co, Inc, Whitehouse Station, NJ, USA) (Cutts et al., 2007). The randomized controlled trials were performed for both the vaccines in over 60, 000 women prior to licensure and were assessed as safe and effective (Agorastos et al., 2009). Both the vaccines are prepared from non-infectious, DNA free virus-like particles produced by recombinant technology and show >90% protection against persisten HPV infection for upto 5 years. However, the vaccines can prevent HPV infection in relatively young women aged upto 26 years and not previously infected with the HPV subtypes covered by the vaccines (Cutts et al., 2007). Ongoing monitoring is necessary to evaluate the safety and efficacy of the vaccines.

Interestingly, although most sexually active women in the general population get infected by HPV, most of these cervical infections are transient, with clearance in 70% to 90% of individuals positive for HPV DNA. In addition, only a small percentage develop long-term HPV infection, which is associated with an increased risk of developing cervical cancer. Thus, an effective host immune response may be an important determinant for the persistence and progression of HPV-induced cervical cancer. Variability in host immunogenetic background is important in determining the overall cellular immune response to HPV infections. Different environmental factors might also play a role in development of cancer of the cervix (Munoz et al., 2002;Parazzini et al., 1998;Plummer et al., 2003;Smith et al., 2003).

1.2 Human Papillomavirus

Papillomaviruses are widely distributed throughout the animal kingdom. These are small non-enveloped viruses with a double stranded DNA genome of approximately 8000 bp (Fig. 1.5) (Doorbar, 2006). The genome consists of a long control region (LCR), an early region (E) and a late region (L). Different types differ by 10% in their sequences and sub-types differ by between 2% and 10% and variants differ by less than 2% (de Villiers et al., 2004).

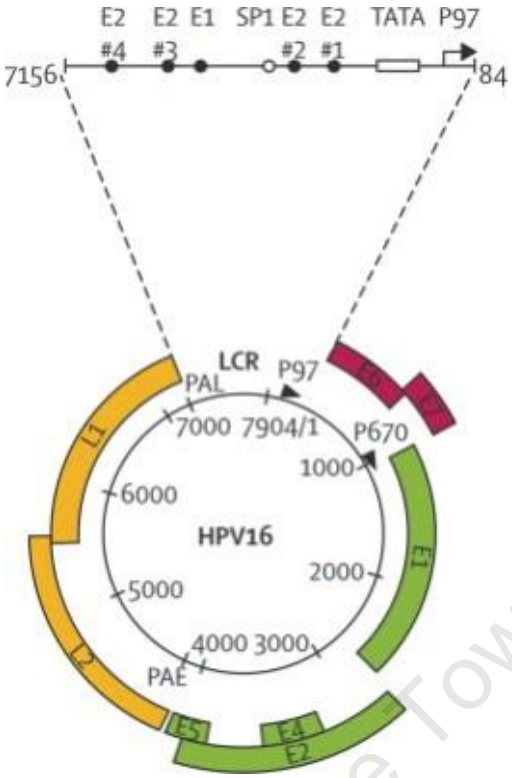

Fig. 1.5: A schematic diagram showing the whole HPV genome (Doorbar, 2006)

1.2.1 Human Papillomavirus types

More than 100 different HPV genotypes have been fully cloned and sequenced (Stoler, 2000) and the origin of the virus is estimated to be before the devepoment of *Homo sapiens (Ong et al., 1993)*. Oncogenic HPVs are the cause of more than 99% of all cervical cancer cases. The association of HPV's with cervical lesions have led to a classification of the virus as 'low-risk' and 'high-risk' strains; the persistence of infection by high risk strains being associated with higher risk for abnormal pap smear (Ho et al., 1998) and invasive carcinoma (Wallin et al., 1999). HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81 are low-risk viruses and HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 are high-risk viruses (Cogliano et al., 2005;Munoz et al., 2003). The five most frequent HPV types among women with ICC in the world are, HPV 16, 18, 33, 45 and 31 according to ranking order while

the five most frequent types in sub-Saharan African women in ranking order are, HPV 16, 18, 45, 33 and 35 (HPV Information Centre, 2009). HPV 16 is predominant in squamous cell carcinomas and HPV 18 in adenocarcinomas. HPV 16 and 18 constitute 70.1% of all the invasive cervical cancer cases across the globe (Castellsague X et al., 2007).

1.2.2 Human Papillomavirus infective cycle and host immune evasion

HPV infects basal keratinocytes of epithelium. Once HPV penetrates inside the cell, it may persist in a latent state (Fig. 1.6) (Stanley, 2008).

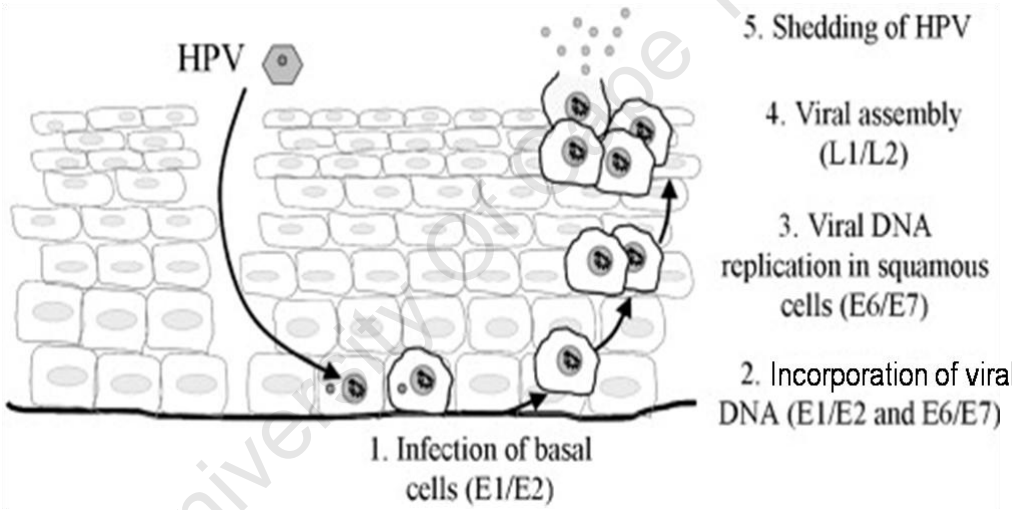

Fig. 1.6: A schematic diagram showing the infective cycle of HPV (Stanley, 2008)

The HPV genome incorporates into the host cell and becomes established as extra-chromosomal elements in the nucleus of the cell and the copy number is increased to 50-100 copies per cell (Paavonen, 2007). As the infected basal cells differentiate and mature, the HPV genome replicates too and the viral DNA is passed on to both daughter cells. One daughter cell migrates away from basal layer and initiates a programme of differentiation while the other one continues to divide in the basal layer (Stubenrauch and Laimins, 1999). Using the host cell the virus expresses its

proteins. The early proteins E6 and E7 immortalise epithelial cells (Halbert et al., 1991;Hawley-Nelson et al., 1989) and promote cell proliferation. This delays natural maturation and differentiation of epithelial cells so as to give the virus time to expand the infection and spread to adjacent cells. While E6 and E7 genes are consistently expressed the other viral gene expressions are disturbed (Baker et al., 1987). At this stage the virus exploits the host cell's transcriptional machinery to synthesize its late proteins L1 and L2 which help the newly formed virus to assemble (Paavonen, 2007). Once an infection is established in the basal cells the virus moves within differentiating cells to the epithelial surface and is shed off and is available to spread. As a result of disrupted cell division, infected epithelial cells outgrow non-infected cells resulting in a dysplasia or tumor. It is speculated that HPV spread to a new host by direct skin contact, possibly within days of viral particle formation.

The integration of the HPV genome into the host cell chromosome is an important event in the development of cervical cancer from pre-cancerous lesions. Changes in the expression of HPV genes at the integration sites may participate in cancer development (Yu et al., 2005). Integration often results in loss of E2 and deregulation of E6 and E7 expression (Doorbar, 2006). These are critical events for the growth of cervical cancer cells. Due to the less differentiated cells, viral genome amplification occurs closer to the epithelial surface in HSIL compared to in condylomas and LSIL. For the same reason the expression of the viral coat protein is also retarded in HSIL (Middleton et al., 2003). Integrated HPV DNA is found in most invasive cancers, high-grade lesions but can also be found in some CIN1 lesions (Fujii et al., 2005). The high-risk HPV E7 protein acts more efficiently than regular E7 protein as a mitotic mutator and increases the chance of error in each

round of cell division (Duensing and Munger, 2004). The high-risk HPV E6 proteins bind to p53 with a better affinity than the low-risk E6 protein and stimulate p53 degradation (Huibregtse et al., 1993). The loss of p53-mediated DNA damage response is one of the most important molecular mechanisms that leads to cervical cancer. Additional genetic changes accumulated over a period of time following the initial HPV infection speeds up the development of the cancer of the cervix.

Cervical carcinoma is accompanied by genomic instability and choromosomal aberrations. While low-risk HPV E6/E7 proteins are not capable of inducing centrosome abnormalities, high-risk HPV E6/E7 proteins can induce centrosome abnormalities and cause genomic instability (White et al., 1994). The key function of HPV E6 and E7 proteins is to cause inactivation of the p53 and pRB tumour suppressor pathways consecutively. Inactivation of p53 by E6 protein suppresses two major events, 1) Cell growth arrest in G1 phase followed by DNA repair and 2) Apoptosis, resulting in DNA damage and survival of infected cells (Linke et al., 1996). The pRB protein acts as a break point for cells progressing into S-phase. Inactivation of pRB allows tumour cells to enter into S-phase leading to cell replication (Dyson et al., 1989).

1.3 Immune response to Human Papillomavirus

The human defense against pathogenic micro-organisms can be divided into early reactions of innate immunity and later responses of adaptive immunity.

1.3.1 Innate immune response to Human Papillomavirus

This is the primary and rapid response of the body to a foreign microbe. Physical and chemical barriers like epithelia, phagocytic cells like macrophages and neutrophils, natural killer (NK) cells, blood proteins including complements and cytokines are the main components of this immune reaction (Bruce A et al., 2002).

During most of the HPV infectious cycle, a wide range of different cytokines, important for dendritic cell (DC) activation and migration and essential signal to kick start the immune response in squamous epithelia such as proinflammatory cytokines, growth factors and chemokines are secreted in low levels by cervical keratinocytes (Stanley et al., 2007). A vital cytokine tumour necrosis factor (TNF) shows a direct antiviral and antiproliferative effect. Apoptosis of the HPV-infected cells and spontaneous regression of papillomas correlate with high levels of expression of TNF-α in infiltrating mononuclear cells (Hagari et al., 1995). TNF-α and another antiviral cytokine interleukin (IL)-1 have both shown an ability to downregulate HPV E6 and E7 gene expression and retard the growth of cell lines harboring the viral genome (Kyo et al., 1994). HPV-infected keratinocytes activate the powerful antiviral defence system of type 1 Interferons (IFNs) consisting of IFN-α, -β and -γ. The type 1 IFNs have antiviral, antiproliferative, anti-angiogenic and immnostimulatory properties that act as a bridge between innate and adaptive immunity and also acrtivates immature DCs (Le and Tough, 2002). High-risk HPV infection down-regulates IFN-α mediated gene expression and the HPV E6 and E7 oncoproteins interferes directly with IFN signalling pathways (Woodworth, 2002). HPV E6 and E7 proteins bind to interferon regulatory factor-3 (IRF-3) and IRF-1 respectively and inhibit IFN-α mediated signal transduction (Arany et al.,

1995;Ronco et al., 1998). NK cells are important components of the innate immune response to HPV infections. NK cells are a sub-set of lymphocytes that kill virus-infected or tumour cells that lack surface expression of MHC class I molecules (Stanley, 2009).

1.3.2 Adaptive immune response to Human Papillomavirus

Adaptive immunity consists of humoral immunity and cell-mediated immunity (CMI). Humoral immunity is mediated by B-lymphocytes and CMI is mediated by T-lymphocytes.

Humoral immunity is mediated by antibodies (Abs) or immunoglobulins produced by B-lymphocytes. Abs are freely circulating in blood and are the principal defence against extracellular microbes. They bind to the foreign particles and help to eliminate them. Abs recognise viral particles in early stages of HPV infection, even though the humoral immune system is not always activated by natural HPV infection. Abs can also neutralize viral particles by binding to their receptors. IgG abs which are specific for HPV epitopes can protect against infection with oncogenic HPV (Nardelli-Haefliger et al., 2003). Studies have shown that higher levels of serum IgG is capable of neutralizing HPV (Nardelli-Haefliger et al., 2003). IgG responses can also promote limited cross-neutralization and cross-protection from different oncogenic HPV types (Einstein, 2008).

Antigen presenting cells (APCs) display antigens derived from intracellular microbes, which are recognised by these cells. The genes in Major Histocompatibility Complex (MHC) encode proteins that perform antigen

presentation. Human MHCs are called Human Leukocyte Antigens (HLAs) and are highly important in inducing effective immune-response against any invading antigens. The HLA genes are localized on chromosome 6. The extraordinary polymorphism of HLA genes suggests 'overdominant selection' or 'heterozygous advantage', presented by individuals heterozygous at HLA loci. A greater variety of antigenic peptides against the invading pathogenic micro-organisms are processed by heterozygotes compared to homozygotes. The highly polymorphic nature of HLAs helps to bind a wide variety of foreign antigenic peptides by heterozygotes compared to homozygotes resulting in protection against different pathogenic microbes. This results in a more efficient immune response against a wide range of pathogens (Doherty and Zinkernagel, 1975;Zinkernagel, 1996). Langerhans cells (LHc) are immature dendritic cells and the resident APC in many epithelial sites of the body, including the cervix. APCs capture antigens and transport them to local lymph nodes where a T-cell response is activated. Studies have reported a reduced number of LHc in genital HPV infection and in SIL (Morelli et al., 1994;Morris et al., 1983;Tay et al., 1987). After capturing antigens the LHc migrate to draining lymph nodes. Cytokines mainly produced by keratinocytes play a helping role in LHc function. IL 1-α, TNF and IL 1-β which activate and promote LHc migration and IL-10 which inhibits LHc migration have been particularly important (Wang et al., 1999). Keratinocytes in normal cervix express TNF-α but low-grade and high grade squamous intraepithelial lesions (LSIL and HSIL) have shown an absence of TNF-α expression (Mota et al., 1999). Cytokine IL-10 was also found absent in normal epithelium and found up-regulated in low and high-grade CIN lesions (Mota et al., 1999).

CMI is mediated by T-lymphocytes which can be classified as CD4+ helper T-lymphocytes and CD8+ cytotoxic T-lymphocytes (CTLs). CD4+ helper T cells can be divided into TH1 and TH2 cells. TH1 cells stimulate responses from macrophages, NK cells and CD8+ CTLs especially during infections of intracellular microbes. The signature cytokine of TH1 cells is IFN- γ but they also produce TNF and IL-2. Many studies suggest that HPV infection triggers a TH1 response. The signature cytokines of TH2 cells are IL-4 and IL-5 but they also produce IL-10 and IL-13 (Mosmann and Sad, 1996). TH2 cells mostly activate humoral immunity. Studies have found low density of TH1 cells and high density of TH2 cells in HSIL compared to normal epithelium (Al-Saleh et al., 1998). It has been shown that HPV specific CD4+ cells in lymph nodes of cervical cancer patients produce IL-10 and suppress HPV specific cell mediated immunity which gives an explanation for a failed HPV specific CD4+ immunity in cervical cancer patients (van der Burg SH et al., 2005). Even though HPV generally elicits a TH1 response, a shift toward TH2 response is associated with HPV linked cervical pathology.

Cytotoxic T Lymphocytes (CTLs) are CD8+ cells that recognise specific viral antigens (or more accurately a small part of the antigen called, “epitope”) and kill virus infected cells by releasing cytotoxins and cytokines. The CTLs control tumour cells through two major pathways, the cell death pathway (Fas-FasL) and the granule secretory pathway. Apoptosis can be induced by any of these pathways. The activation of Fas-FasL cell death pathway in induction of apoptosis is described in detail later in this section.Apoptosis induced by the granule exocytosis pathway is regulated by Perforin (PFN) and granzyme (gzm) molecules. Once a target cell is engaged, cytoplasmic granules of the cytotoxic cell migrate toward the contact site

and fuse with the plasma membrane releasing granule contents. These granules diffuse toward the target cell and granzymes are delivered which is facilitated by perforin. Granzymes are encoded by five gzm genes in humans namely, *gzmA*, *gzmB*, *gzmK*, *gzmH* and *gzmM*. PFN plays a critical role with gzmA and gzmB in the control of viral infections (Russell and Ley, 2002). However, their importance in eliminating tumour cells is poorly understood (Revell et al., 2005). The understanding of the biological roles played by other gzms, termed as orphan gzms (gzmC-G, gzmK, gzmL, gzmN and gzmM) is also not clear.

Apotosis in virus infected cells is induced by gzmA (Hayes et al., 1989) and gzmB (Shi et al., 1992) in presence of PRF. Studies with *ex vivo* derived Tc cells from gzmKO mice showed impaired apoptosis in the absence of gzmB and more so in the absence of both gzmA and gzmB (Pardo et al., 2002). The perforin/granzyme pathway plays an important role in controlling HPV infection. *In vitro* Studies have shown increased anti-tumour activity of HPV E-7 specific CTLs (Sin et al., 2009) in destruction of HPV infected keratinocytes.

Activated CTL and HPV 16 E6 and E7 specific CTL have been found in SIL and HPV specific CTL in individuals with a previous or ongoing HPV infection (Alexander et al., 1996;Bontkes et al., 1997;Evans et al., 1996;Evans et al., 1997;Nakagawa et al., 1997). A longitudinal study of women with HPV 16 infection showed lack of CTL response to HPV E6 but not to HPV E7 protein correlated with persistent HPV infection, suggesting that a CTL response to HPV 16 E6 protein is important for the clearance of HPV and for neoplastic progression (Nakagawa et al., 2000). In summary, CTLs are the main mediators of HPV clearance and CD4+

helper T-cells help to maintain antiviral CD8+ T-cell activity for the generation of strong virus neutralising Abs.

1.4 Environmental risk factors

Along with high-risk HPV infection there are some additional risk factors that have been studied and have been reported to play an important role in the outcome of cervical cancer. Studies have indicated cervical dysplasia, smoking (Plummer et al., 2003), high parity (Munoz et al., 2002) and long term use of oral contraceptives (Moreno et al., 2002;Smith et al., 2003) and high number of sexual partners as the risk factors for persistent HPV infection. Co-infection with other sexually transmitted agents such as Herpes Simplex Virus-2 (HSV-2) (Castellsague et al., 2006;Hildesheim et al., 1991;Smith et al., 2002) might also add to the risk.

1.5 Role of host genetic risk factors in cancer

Cancer can be termed a class of diseases where the main feature is uncontrolled cell growth due to genetic abnormalities occurring either on cancer suppressing and cancer promoting genes. Genetic changes in regulatory mechanisms alter normal cell behaviour and cause cellular abnormality (Kinzler WK and Bert V, 2002). There are 3 major classes of genes affecting carcinogenesis; tumour suppressor genes, oncogenes and DNA repair genes. Tumour suppressor genes inhibit cellular uncontrolled cell proliferation while oncogenes, such as growth and transcription factor genes (like HA-RAS) generally promote cell proliferation. Mutations in these genes result in either loss or gain of activity, respectively (Knudson, 2001). Under

normal circumstances cells possess mechanisms (e.g DNA repair genes) to maintain the genome in areas where mutations have occurred thereby restoring the cells to their normal genotype or programming them to apoptose and get removed from the system. A driving force of tumour development is genetic instability i.e. higher frequency of mutations. The genetic instability can be caused by defects in DNA repair systems which are a result of mutations in DNA repair genes. As apoptosis is an important factor for regulating uncontrolled cellular growth, abberant functioning of genes involved in apoptotic pathways (e.g. Fas, FasL) can lead to cancer development. Genes encoding cytokine proteins (like, TNF, IL, IFN) play a crucial immunological role in protection against pathogenic microbes and are of particular interest for viral infection leading to cancer. Human leukocyte antigen (HLA) genes are also important in determining host immune response to pathogenic organisms due to their highly polymorphic nature. Almost all cancers are caused by abnormalities in the genetic material that can be due to randomly acquired errors in DNA replication or inherited (Croce, 2008).

1.6 Host genetic risk factors for cervical cancer

The genetic link to cervical cancer development is supported by epidemiological studies. A hereditary component of cervical tumours has been indentified by the comparisons of twins (Ahlbom et al., 1997) and in a mother-daughter family studies (Hemminki et al., 1999). The possibility of host genetic predisposition is strengthened by the observation that biological first degree relatives of a woman who has developed cervical tumour, experience a two-fold risk of developing cervical tumour compared to non-biological relatives of women with cervical tumour

(Magnusson et al., 1999). The heritability of cervical tumours have been determined as 27% (Magnusson et al., 2000).

Three different strategies can be used for genetic studies of human complex diseases, 1) Family studies, 2) Animal models and 3) Case-control studies. As it is difficult to find families or relatives affected by the same disease and also to find animal models, case-control studies are hugely popular and are mostly carried out by researchers for the genetic studies of different complex diseases.

There are two main approaches for investigating the genetic associations of complex and quantitative traits, genome-wide scan and candidate gene approach. Each of these approaches has specific advantages and disadvantages. Genome-wide scan is a method to search the entire human genome to find the causative genetic associations for a particular disease. This is done without any presumptions of the importance of functional features of the trait under investigation. Several DNA markers are used in family-based or experimental based designs on a large number of candidate genes. The main disadvantage of this method is the sheer expense the intensive resources to carry out the work. The candidate gene approach is based on the existing knowledge from the literature. The candidate genes are generally the genes with known biological function directly or indirectly affecting the trait under investigation. This approach is comparatively less expensive and can be done with limited number of resources (Zhu and Zhao, 2007)**.** Due to these advantages candidate gene approach is highly popular and has been extensively applied for gene-disease research, genetic association studies, biomarker and drug target selection in different organisms from animal to humans (Tabor et al., 2002). The main limitation of traditional candidate gene approach is its dependence on known or presumed biology of the investigated

phenotype. Low replication of results and its limited ability to include all possible causative genes are other disadvantages of this approach (Tabor et al., 2002).

Candidate gene approach was adapted for the present investigation mainly due to the well known biology of persistent HPV infection and cervical cancer. The host genetic association studies with cervical cancer in different population led us to a better understanding of the pathways to be investigated. The lack of host genetic association studies with cervical cancer in African population had set a good platform for a candidate gene approach to investigate the host genetic associations of cervical cancer.

Several different candidate genes have been investigated by different groups across the globe. Some of the major host genes known to influence the outcome of the disease (other than the candidate genes investigated in this thesis which will be discussed in detail later in this chapter) are reviewed below.

HLA DQ and DR genes have been extensively studied by many groups in different population across the globe. DQA1*01, *03 and DQB1*02, *03, *04, *06 alleles have been associated with increased risk of developing the disease. A protective association has also been found with DQA1*0501 and DQB1*0201, *0103, *0301/*0501, *04, *05, *050201, *06 alleles. A protective effect of the DRB1 *13/DQB1 *0603 had been the most consistent HLA findings of all (Hildesheim and Wang, 2002).

The *Tp53* codon-72 (Arg/Pro) polymorphism was first investigated by *Storey et. al.* in cervical cancer patients (Storey et al., 1998). They reported a seven times higher risk of developing HPV-associated cervical carcinogenesis in individuals with Arg homozygosity. Many publications investigating the *Tp53* codon-72 polymorphism in cervical cancer susceptibility in different ethnic groups followed with contradictory results (Jee et al., 2004).

Tumour Necrosis Factor (TNF) is a cytokine and is produced mainly by activated macrophages. Several polymorphic sites have been reported in *TNF* locus including five microsatellites of TNFa-e. *TNF-a* is closely linked to *TNF-β* gene and contains 14 different alleles (a1-a14) with an AC/GT dinucelotide repeat. Several groups investigated the role of various polymorphic sites of *TNF* gene in association to cervical cancer in different ethnic populations. A positive association was found with cervical cancer and the presence of *TNFa-8, TNF-α -572, -857, -863* and also with *TNF G-308A* (Zoodsma et al., 2005). *TNFG-308A* did not show any association in South African population (Govan et al., 2006).

MHC class I polypeptide-related sequence A or *MICA* genes are expressed by keratinocytes and epithelial cells on the cell surface and interacts with T cells with an important role in immune response and direct induction of mucosal immunity. Exon 5 of *MICA* gene contains a microsatellite locus with 5 alleles, in strong linkage disequilibrium with *HLA-B* alleles. Different polymorphic sites of *MICA* were investigated but none were found to have an association with the severity of the disease (Zoodsma et al., 2005).

Any changes in *WAF1/p21* gene may affect the regulation of cell growth and result in excessive proliferation of cancer cells. A serine to arginine change has been well documented followed by a C to A transversion at the third base of codon 31. Different groups studied this polymorphism with mixed results. Ser/Ser genotype was found to be the susceptible genotype for adenocarcinoma in high risk HPV but not for squamous cell carcinoma. Ser/Arg genotype was associated with susceptibility to cervical cancer (Zoodsma et al., 2005).

IL-10 is a TH2 type cytokine produces a suppressive effect on cell mediated immunity. One of the polymorphisms that is associated with low, medium or high production of *IL-10* situates in the promoter region of the gene at position -1082. Several studies investigated this polymorphism and reported conflicting results. -1082 A/G was found to be the risk genotype for cervical cancer (Zoodsma et al., 2005).

Methylenetetrahydrofolate reductase or MTHFR enzyme regulates the metabolism of folate and methionine, which are critical for DNA methylation and synthesis. C to T transition at nucleotide position 677 of the *MTHFR* gene results in alanine to valine. Compared to homozygous wild type (Ala/Ala), both heterozygous (Ala/Val) and homozygous mutant (Val/Val) variants reduce MTHFR enzyme activity in individuals with a low folate status. Hence this polymorphism might cause an abnormal DNA methylation and DNA synthesis which can lead to an increased risk of developing cancer. This polymorphism is less frequent in blacks than in Caucasians. Different groups investigated the effect of this polymorphism in different population. Conflicting results were

found by different groups. Some found the polymorphism as a risk factor to develop cervical cancer while others did not (Zoodsma et al., 2005).

1.7 Genes investigated in this thesis

In this thesis we are focusing on the common polymorphisms in some of the major host genes with important immunological functions. Three genes in the cell death pathway, namely Fas, FasL and CASP8 and an inflammatory pathway gene CCR2 were focused on for this thesis. The effects of the common polymorphisms on these four genes on susceptibility to infection with HPV leading to cervical cancer and HSV-2 infection were investigated.

1.8 Fas and Fas Ligand (FasL) genes and their roles in immune response

The Fas gene is situated on chromosome 10q24.1 and the FasL on 1q23. Fas and FasL are two crucial genes on the cell death pathway. Fas is a cell-surface death receptor also called CD95. Binding of the Fas receptor (FasR) with FasL triggers expression of caspase-8, which in turn signals the cells to initiate apoptosis (Fig. 1.7) (Ando et al., 1997;Cotran RS et al., 1999;Zhang et al., 2004).

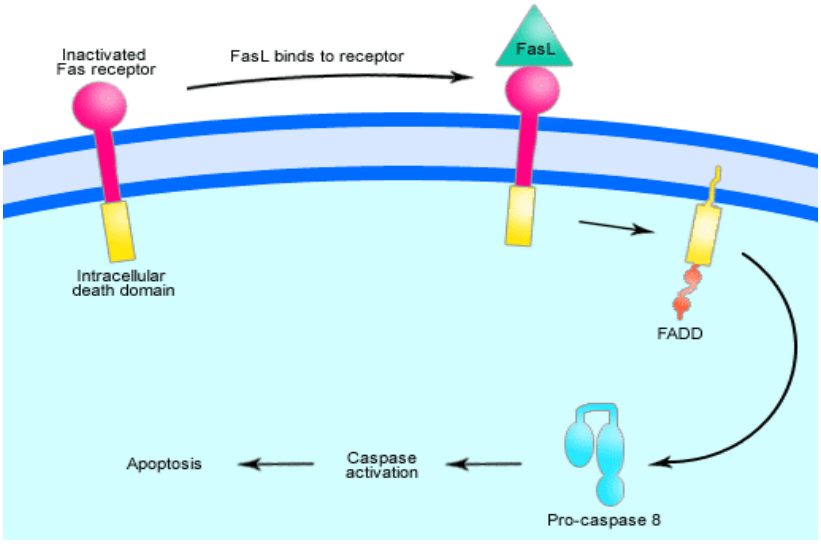

Fig. 1.7: Activation of Fas-FasL pathway (Cotran RS et al., 1999)

Apoptosis is a highly regulated process of cell suicide, also called as programmed cell death. The role of corrupted apoptosis has been well documented in the development of tumorigenesis, a process by which normal cells transform into tumour cells. Tumorigenesis is achieved not only by increased cell proliferation but also by a decreased apoptotic rate (Zornig et al., 2001). Defects in genes that paly a role in the mechanism of apoptosis such as Fas and FasL has been identified as cancer causing event.

The signal produced by the DNA binding of transcription factors SP1 (stimulatory protein 1) and STAT1 (signal transducer and activator of transcription 1) are associated with transcriptional activation and expression of Fas gene. Fas -1377 G>A and -670 A>G are situated within SP1 and STAT1 binding sites respectively. It has been shown that the substitution of G by A at position -1377 of the Fas promoter region considerably reduces SP1 binding (Huang et al., 1997;Sibley et al., 2003) thus affecting the regulation of apoptosis. Gamma interferon activation signal (GAS) is a binding element responsible for DNA binding of STAT1. -670 A>G SNP is located

within GAS binding site. The presence of G at position -670 in the Fas promoter region partially or completely abolishes the GAS element, hence significantly reducing the Fas gene expression (Huang et al., 1997;Kanemitsu et al., 2002;Sibley et al., 2003) which results in decreased activation induced cell death (AICD) of virus-infected target cells leading to uncontrolled growth of the virus. It has also been shown that a higher basal expression of FasL is associated with the C allele at position 844 of the FasL gene (Wu et al., 2003). Reduced expression of FasL inhibits the apoptotic activity of the Fas-FasL pathway.

These Fas and FasL polymorphisms that impair the apoptotic signals have been associated with susceptibility to a number of different cancers such as, malignant lymphomas, lymphoproliferative syndrome, T-cell lymphoma, Hodgkin's disease, urinary bladder cancer, lung cancer, esophageal squamous-cell cancer (Davidson et al., 1998;Lee et al., 1999;Peters et al., 1999;Sun et al., 2004;Takahashi et al., 1994;Zhang et al., 2005), and cervical cancer (Dybikowska et al., 2004;Engelmark et al., 2004;Ivansson et al., 2007a;Lai et al., 2003;Lai et al., 2005;Sun et al., 2005). Six studies have shown conflicting results on the role of Fas and FasL polymorphisms but this could be due to the fact that the studies were done in different populations. In one group the FasR-670A allele and A/A genotype were associated with higher risk of developing cervical cancer (Lai et al., 2003). The same group replicated the results for FasR-670 in another case-control study (Lai et al., 2005) and reported no role for Fas-1377 G→A or FasL844 T→**C** polymorphisms with disease severity. Two other groups found different results for FasR-670, in a case control study (Dybikowska et al., 2004) and in an affected sib-pair (ASP) study (Engelmark et al., 2004). Lastly, the two other groups, studying Chinese subjects (Sun et al., 2005) and

Swedish subjects (Ivansson et al., 2007a), respectively, reported an association of FasL844C allele and FasL-844C/C genotype with susceptibility to cervical cancer.

Fas and FasL can be expressed in a variety of normal human tissues. Although some studies investigated the correlation of Fas and FasL expression between malignant and normal tissues, the results are still not clear (Jiang et al., 2002). A recent study showed Fas expression in cytomembrane (CM), cytoplasm and cytoblasts. Fas was basically expressed in four target cells, gastric epithelial cells (GECs), gastric carcinoma cells (GCCs), normal gastric stroma-infiltrating lymphoid cells (NGILs) and tumour-infiltrating lymphoid cells (TILs). The expression of Fas was significantly higher in GECs compared to GCCs and in TILs compared to NGILs (Liu et al., 2009). The FasL was also expressed on the CM, cytoplasm and cytoblasts. FasL expression was seen in all the four target cells like Fas, GECs, GCCs, NGILs and TILs. There was no significant difference in FasL expression between GECs and GCCs or NGILs and TILs (Liu et al., 2009).

1.9 CASP8 gene and its role in immune response

The *CASP8* gene is localized on chromosome 2q33 and codes for Caspase 8 protein which is involved in initiating apoptosis. This gene is also known as, *FLICE* or *MCH5*. Various caspases are found downstream of Fas and FasL, among which caspase-8 plays one of the most important roles in generating apoptotic signals (Siegel, 2006). It is central in activating a caspase-cascade receiving apoptotic signals from Fas-FasL interaction which triggers apoptosis (Ju et al., 1995). Mammalian cells can go through apoptosis by two principle pathways, i.e. the

receptor (extrinsic) and the mitochondrial (intrinsic) pathway (Fulda and Debatin, 2006). Stimulation of any of the pathways can lead to the activation of caspases (Degterev et al., 2003). Apoptosis, one of the most crucial functions of the human immune system to control tumours by eliminating potentially malignant cells is a form of programmed cell death to maintain homeostasis in many tissues (Evan and Vousden, 2001). Apoptosis is a mechanism of restricting uncontrolled growth of cells. Thus reduced apoptosis would lead to cancer formation and progression (Lowe and Lin, 2000). One of the main characteristics of cancer is to bypass apoptosis by forcing the cell to ignore the apoptotic signals that in a normal circumstance would lead to cell suicide (Hanahan and Weinberg, 2000). Signals generated by the interaction of cell-surface death receptor Fas together with Fas ligand (FasL) activates caspase leading to apoptosis (Fig. 1.8) (Harvey Lodish, 2003;Siegel, 2006;Suda et al., 1993).

The expression and activities of caspases have been shown by several studies as one of the most important factors for survival of immune cells and have also been associated with many pathological conditions including cancer (Siegel, 2006). Apart from apoptosis caspase-8 also has various other functions such as controlling T cell proliferation (geciras-Schimnich et al., 2002;Launay et al., 2005) and differentiation and proliferation of B cells and NK cells (geciras-Schimnich et al., 2002;Launay et al., 2005).

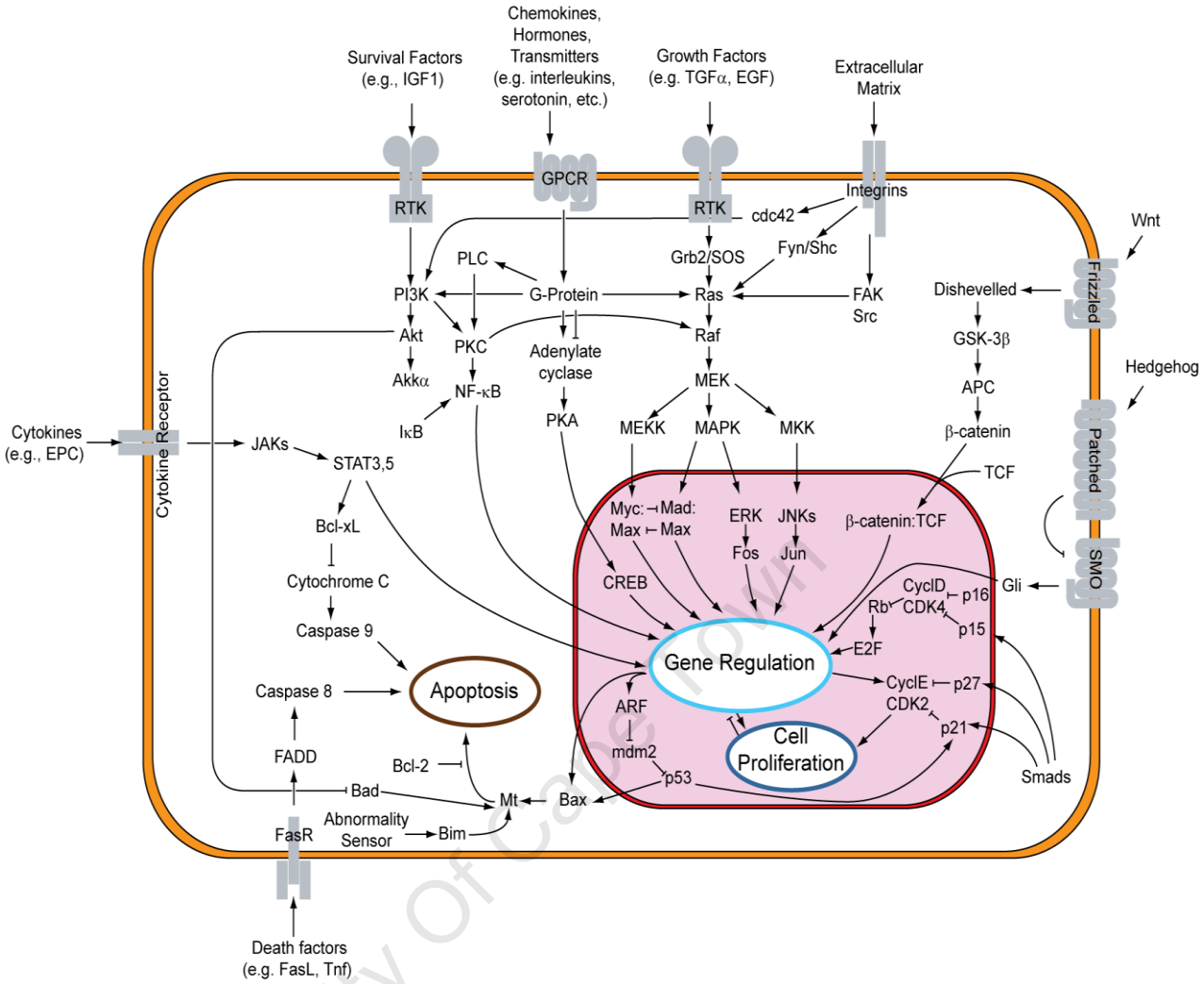

Fig. 1.8: A detailed diagram showing the activation of Caspase-8 through Fas-FasL pathway (Harvey Lodish, 2003)

Subcellular localization of active caspase-8 may control either proliferation or apoptosis (Koenig et al., 2008). Polymorphic variations in *CASP8* gene might result in differential expressions of caspase-8 leading to altered apoptosis and eventually influence the tumour progression and susceptibility to cancer.

Two commonly known polymorphisms in the *CASP8* gene have been well studied, namely *CASP8* D302H and *CASP8* -652 6N ins/del (Sergentanis and Economopoulos, 2009). A functional polymorphism of a six nucleotide deletion of AGTAAG at the position 652 of the promoter region of the *CASP8* gene (*CASP8* -

652 6N ins/del) has been identified. This six nucleotide deletion destroys a binding element for transcriptional activator stimulatory protein-1 (SP1) which decreases capase-8 expression leading to reduced apoptosis (Sun et al., 2007). This polymorphism has been associated with several pathological conditions including multiple types of cancers including cervical cancer (Cybulski et al., 2008;De et al., 2009;Frank et al., 2008;Ji et al., 2009;Li et al., 2008;Ni et al., 2009;Pittman et al., 2008;Sun et al., 2007;Wang et al., 2009;Yang et al., 2008). *CASP8* -652 6N del/del genotype was shown to reduce the risk of lung cancer, esophageal cancer, gastric cancer, breast cancer and colorectal cancer in a Chinese population (Sun et al., 2007). Studies among Germans (Frank et al., 2008), Polish (Cybulski et al., 2008) and Italian subjects (De et al., 2009) did not find any association of this variant with breast cancer risk. However, this variant was associated with later age at diagnosis for breast cancer in Italian population (De et al., 2009). The association with colorectal cancer was also not found in a British population (Pittman et al., 2008). *CASP8* -652 6N del/del genotype was reported to decrease the risk of cutaneous melanoma in an American population (Li et al., 2008) and pancreatic (Yang et al., 2008) and bladder cancer (Wang et al., 2009) in Chinese population. The variant had no effect on prostate cancer in a Polish population (Cybulski et al., 2008). *CASP8* -652 6N del/del genotype has also been associated with decreased sperm apoptosis and poor sperm motility (Ji et al., 2009) and with increased risk of Coal Worker's Pneumoconiosis (Ni et al., 2009) in Chinese population.

Only one study investigated the influence of *CASP8* polymorphism on development of cervical cancer (Sun et al., 2007). Sun T et. al. studied the role of *CASP8* -652 6N

ins/del polymorphism in a Chinese population and reported a reduced risk of developing cervical cancer with *CASP8* -652 6N del/del (Sun et al., 2007).

1.10 Chemokine receptor-2 (CCR2) gene and its role in immune response

CCR2 gene is situated on chromosome 3p21. One of the earliest responses of human body to injury or infection is the release of chemokines that triggers penetration of local inflammatory and immune cells (Fig. 1.9) (Balkwill, 2004;Kleine-Lowinski et al., 2003).

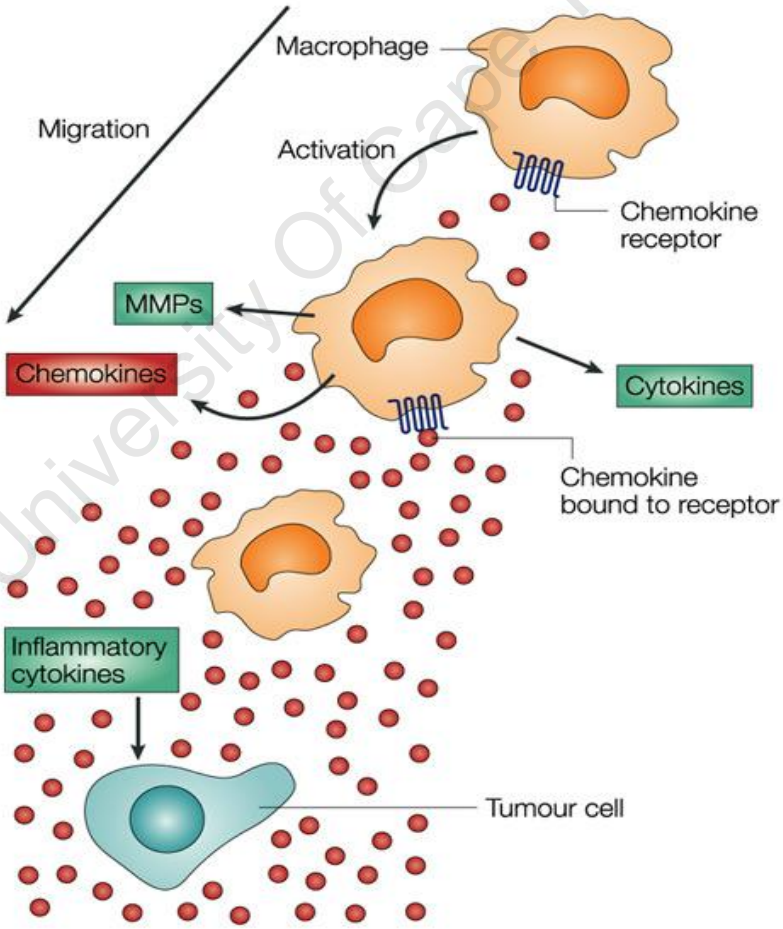

Fig 1.9: A diagram showing chemokine activation following inflammation and recruitement of macrophages (Balkwill, 2004).

Chemokines are chemoattractant cytokines that regulate migration of leukocytes by binding to G-protein coupled cell-surface receptors. It has been postulated that HPV disrupts the interaction between epithelial cells and the immune system by deregulating the expression of chemokines, mediated by (HPV) E7 that interacts with IRF-1 (Gerard and Rollins, 2001) which have been associated with bacterial or viral infections, autoimmune diseases, heart diseases and many others (Gerard and Rollins, 2001). Chemokine receptor 2 or CCR2, has affinity for CCL2 (monocyte chemoattractant protein-1 (MCP-1)), CCL7, CCL8 and CCL13 ligands and is expressed on basophils, monocytes, dendritic cells, activated T-cells and NK cells. CCR2 is a major receptor for the MCP-1 which is produced largely by tumour cells (Kurihara et al., 1997;MacKay, 2001) and is responsible for recruiting macrophages to tumors in bladder, cervix, ovary, lung and breast. The role of macrophages in tumour development and control is multifactorial. In early stages, macrophages have tumour cytotoxic characteristics but once tumour cells have evaded the immune system, the macrophages, switch to play a role in tumour angiogenesis (Lamagna et al., 2006;Lin and Pollard, 2007;Vicari and Caux, 2002). Though macrophages display tumor cytotoxicity, tumor-associated macrophages (TAMs) mainly have protumor functions (Mantovani et al., 1992) and help in tumor angiogenesis. More expression of MCP-1 recruits more macrophages and speeds up the process of tumor progression. It was shown that 1/6 high grade squamous intraepithelial neoplasia expressed MCP-1 compared to 4/5 squamous cell cervical caracinomas (Riethdorf et al., 1996) suggesting that MCP-1 plays a greater role in invasive cancers compared to pre-cancers. This was further confirmed by *in situ* hybrisation studies (Kleine-Lowinski et al., 1999) where MCP-1 mRNA expression was detected in the stroma surrounding the tumour particularly at the invasion front. Tumor cells have been

reported with high levels of MCP-1 expression (Kleine-Lowinski et al., 1999). Macrophages which are recruited by MCP-1 chemokine, express CCR2 on their cell-surface.

CCR2 has two isoforms – CCR2A and CCR2B originated from the CCR2 gene by alternative splicing. A single nucleotide polymorphism (SNP) of G to A at position 190 of CCR2 gene changes amino acid valine (GTC) to isoleucine (ATC) at codon 64 (*CCR2-V64I*). This conservative amino acid change takes place in the first transmembrane domain of CCR2A and CCR2B. This change makes CCR2A more stable and increases its half-life but does not any way affect the stability of CCR2B isoform. The increased stability of CCR2A might lead to accumulation of a large amount of this isoform on the macrophage cell-surface which also interferes with the CCR2B function. This misleads the cells and the macrophage recruitment drops during the development of the tumor which hampers the tumor angiogenesis and eventually reduces the risk of progression to tumour.

This polymorphism (*CCR2-V64I*) has been extensively studied and several reports show a protective role of the polymorphism with AIDS (Doms and Peiper, 1997;Ioannidis et al., 2001;Mulherin et al., 2003;Smith et al., 1997), multiple sclerosis (Miyagishi et al., 2003) and breast cancer (Zafiropoulos et al., 2004). It is also associated with increased risk of carotid atherosclerosis (Nyquist et al., 2009) and reduction in the risk of renal transplant rejection (Omrani et al., 2008). There are conflicting reports on the role of the *CCR2-V64I* polymorphism in the development or risk of cervical cancer (Coelho et al., 2005;Coelho et al., 2007;Ivansson et al., 2007a;Zheng et al., 2006). A study comparing SIL patients to ICC patients in a

Portuguese population reported that the *CCR2-64I* variant was associated with reduced risk of developing ICC from HSIL (Coelho et al., 2005). The same group conducted a case-control study in the same population comparing women with HSIL to a control group and found the *CCR2-64I* variant was a risk allele for developing HSIL (Coelho et al., 2007). Among two studies in Swedish populations, the first study (Ivansson et al., 2007a) reported that the *CCR2-64I* variant of this SNP was associated with decreased risk of developing cervical cancer. The second Swedish study did not find any association of *CCR2-64I* variant with either HPV infection or cancer of the cervix (Zheng et al., 2006).

1.11 Host genetic studies of cervical cancer in Africa

Most of the host genetic studies of cervical cancer have been done with Caucasian population. Only a handful of studies have been done in Africa (Stanczuk et al., 2001;Stanczuk et al., 2003;Tanara et al., 2003). The IL-10 promoter polymorphism was investigated in a black Zimbabwean population. It was observed that IL-10 -1082 A/G increases the risk to cervical cancer in that population (Stanczuk et al., 2001). The same group investigated the role of TNF-α promoter polymorphism in the same population and showed no association of TNF-α -308 A/G with risk of cervical cancer (Stanczuk et al., 2003). The change of amino acid from proline to arginine at codon-72 of Tp53 gene was investigated in a black Gambian population. No association was found with Tp53 codon-72 polymorphism and risk of cervical cancer in that population (Tanara et al., 2003).

The studies with South African population are also limited compared to elsewhere (Govan et al., 2003;Govan et al., 2006;Govan et al., 2007;Pegoraro et al., 2000;Pegoraro et al., 2002). Most of the host genetic studies of cervical cancer in South Africa were done by our group (Govan et al., 2003;Govan et al., 2006;Govan et al., 2007). Tp53 codon-72 polymorphism was investigated in black South African and mixed-ancestry population (Govan et al., 2007;Pegoraro et al., 2000;Pegoraro et al., 2002). There was no association found with Tp53 codon-72 polymorphism and cervical cancer in black (Govan et al., 2007;Pegoraro et al., 2000) and mixed-ancestry South African populations (Govan et al., 2007). However, Tp53 codon-72 Arg was found in more cervical cancer patients infected with low or intermediate risk HPV in black South African populations (Pegoraro et al., 2002). IFN-γ A/T and IL-10 -1082 A/G polymorphisms were found to have no association with cervical cancer in black and mixed-ancestry South Africans (Govan et al., 2003). TNF-α -308 A/G also did not show any association with cervical cancer in black and mixed-ancestry South African population (Govan et al., 2006).

1.12 Project Motivation

Cervical cancer is the most important cancer in women in developing countries. Given that only a fraction of the HPV-infected women develop cervical cancer over time, it is very important to understand why some women develop the disease while others are able to control the viral infection. HSV-2 infection is a major sexually transmitted disease (STD) and is also a co-factor in the development of cervical cancer. Very little is known about the genetic factors involved in HSV-2 infection

and susceptibility. It is an established fact that host genetics play a role in the outcome of infectious diseases and cancer.

Cervical cancer is a multifactorial disease which is dependent on the complex interactions of different host and viral factors including environmental factors as well. It is evident that individual immune response plays a major role in determining the regress or progress of an HPV infection. Host genes are known to play a vital role in shaping an individuals immune response to a specific viral infection as well as to target malignant cells. This literature review clearly points towards a gap of knowledge regarding the host genetic factors that might contribute to persistent HPV infection leading to pre-cancer lesions and eventually cancer of the cervix. Some contradictory reports on different population have tried to shed light on this field but African populations have mostly been untouched till now.

This thesis aims to understand the effects of specific polymorphisms in genes involved in cell death pathway (i.e. Fas, FasL and CASP8) and in macrophage recruitment (e.g. CCR2) in HSV-2 infection, HPV infection, pre-cancerous cervical lesions and cervical cancer in South African women of indigenous black and mixed-ancestry origin.

As mentioned above in the literature review the prevalence of HPV infection and cervical cancer is high in Southern Africa. This is largely due to poor cervical screening and treatment programs. Therefore poverty and lifestyle factors are regarded as the major reasons for the high incidence of cervical cancer. However, the effects of other factors cannot be ruled out. Environmental and host genetic factors

are known to play vital roles in persistent HPV infection and cervical cancer. In this thesis we explored the roles of functional polymorphisms of some of the major immune response genes and their effects on HPV infection, pre-cancerous lesions, cervical cancer and also on HSV-2 infection, a co-factor for developing cervical cancer. The understanding of the effects of these polymorphisms leading to functional changes on cervical cancer would afford a better understanding of the HPV and cervical cancer biology. The implications of our findings not only open up the understanding of the host immune response to persistent HPV infection leading to cervical cancer and to HSV-2 infection but also help us to understand the difference in host response between different populations.

CHAPTER 2

FAS AND FASL GENE POLYMORPHISMS ARE NOT ASSOCIATED WITH CERVICAL CANCER BUT DIFFER AMONG BLACK AND MIXED-ANCESTRY SOUTH AFRICAN WOMEN

2.1 Introduction

The signal generated by the interaction of cell-surface death receptor Fas along with FasL triggers apoptosis. Apoptosis is one of the most crucial mechanisms of human immune system to eliminate HPV-infected cells. Defects in the cell death pathway due to functional polymorphisms in Fas and FasL genes might influence the effectiveness of the apoptotic mechanism and hence the outcome of HPV infection (the roles of Fas, FasL and their polymorphisms have been reviewed in the literature review). FasR-1377G/A, FasR-670A/G and FasL-844T/C polymorphisms have been associated with several disorders and also with cervical cancer in different populations as described in the literature review. These polymorphisms were not studied in African populations with regards to cervical cancer. This chapter focuses on the roles of FasR-1377G/A, FasR-670A/G and FasL-844T/C polymorphisms in cervical cancer in South African women of black and mixed-ancestry.

2.2 Materials and Methods

Participants

Participants were 447 women with invasive cervical cancer (106 black African and 341 women of mixed-ancestry) and 424 controls (101 black African and 323 women of mixed-ancestry) without cancer of the cervix. Incident cases of symptomatic invasive cervical epithelial cancer (stage 1b-IVb), diagnosed a maximum of six months previously were recruited from Groote Schuur Hospital and Tygerberg Hospital in the Western Cape Province, South Africa. Hospital controls were series matched in a ratio of 3:1 to the cases on decade of age, ethnic group and area of residence (urban/rural). The control women were selected to be representative of the population from which the cases were selected, specifically similar to the cases with respect to age, ethnicity and domicile status (urban/rural), so that case-control status would not be associated with either of those. The participants of indigenous African origin were considered as "black African" and the participants who carried mixed lineages in any of their parental ancestry were considered as "mixed-ancestry". There were 264 (59%) urban cases and 183 (41%) rural cases compared to 247 (58%) urban controls and 177 (42%) rural controls. Participants living in towns or cities (according to the national list of towns and cities) were considered as "urbans" and participants living in villages or countrysides were considered as "rural". The cases and controls formed part of a study to investigate the association of oral contraceptives with cervical cancer (Hoffman et al., 2003;Shapiro et al., 2003).

Clinical specimens

Blood was collected from cases and controls, following written informed consent and stored at -80℃. The study was approved by the University of Cape Town Research Ethics Committee (REC REF: 075/2009). The subject identifiers were permanently unlinked from the specimens. The HIV infection status was known for 98% (n = 437) of the cases and 94% (n = 398) of the controls and 5% of the cases and 4% of the controls were positive for HIV infections. Of the controls 13% were positive for high risk HPV by Digene Hybrid Capture II and 14.5% had abnormal cervical cytology.

Extraction of genomic DNA

The genomic DNA was extracted using TotalNucleicAcid Extraction kit for MagNA Pure Compact nucleic acid extractor (Roche Diagnostics, Germany).

Determination of FasR/FasL polymorphisms

Reactions were conducted in 5 µl volumes and contained 10 ng DNA, 1.75 µl PCR MasterMix (Applied Biosystems), 0.087 µl TaqMan assay mix (FasR-1377 (rs2234767, assay id, C_12123966_10), FasR-670 (rs1800682, assay id, C_9578811_10), FasL844 (rs763110, custom-designed)) (Applied Biosystems) and 1.16 µl distilled water. Cycling conditions on the ABI prism 7900 HT (Applied Biosystems) were 2 minutes at 50˚C, 10 minutes at 95˚C, followed by 40 cycles of 15 seconds at 92˚C and one minute at 60˚C. End point fluorescence was measured immediately after cycling. Alleles were assigned using SDS 2.0 software (Applied Biosystems) (Zoodsma et al., 2005) (this work was carried out at Uppsala University, Sweden). Two figures have been shown below with the best (Fig. 2.1) and the worst

(Fig. 2.2) genotype clusters of TaqMan assay measured. The undetermined samples were repeated for genotyping at the end.

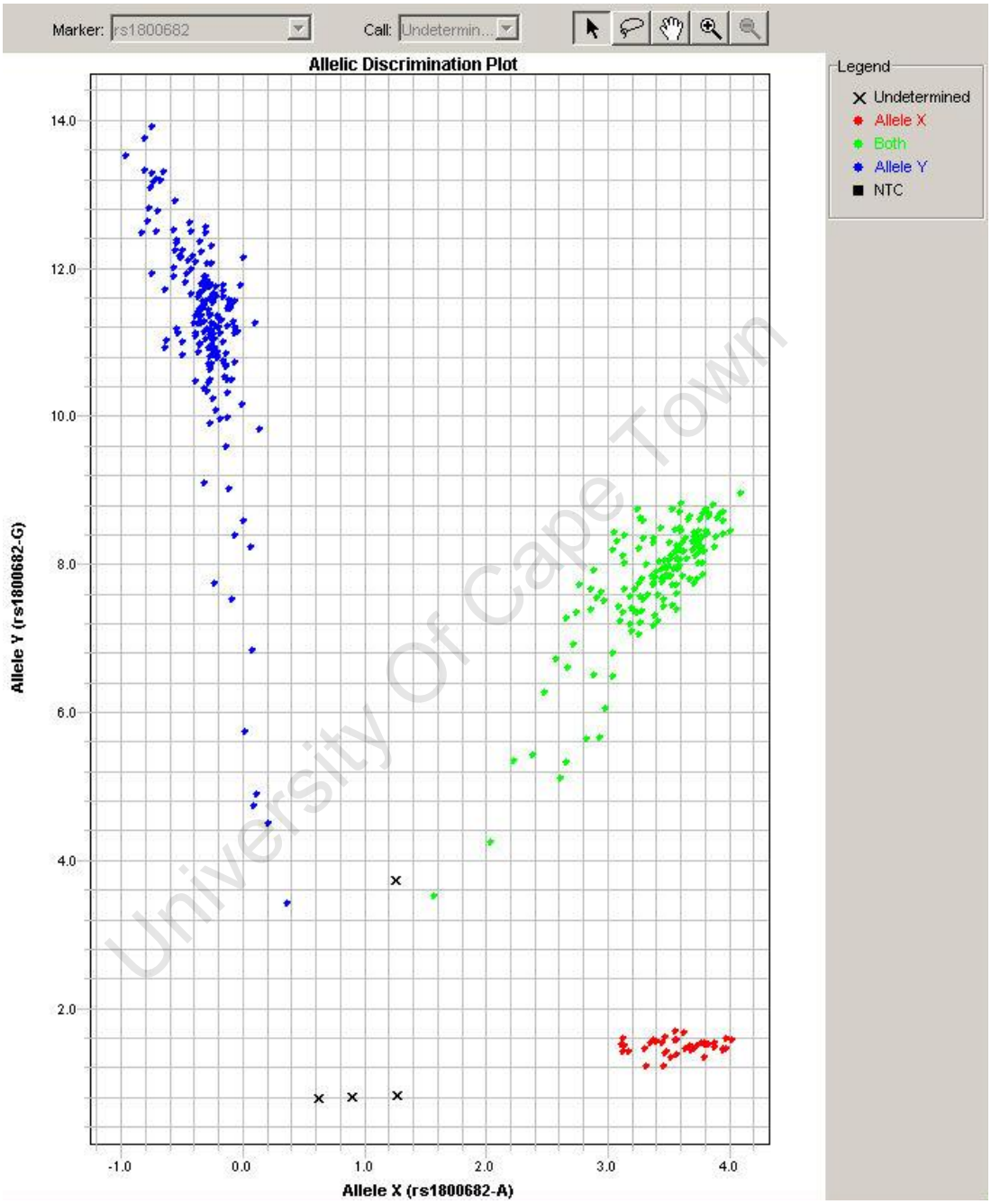

Fig. 2.1: One of the genotype clusters for TaqMan assay showing the distribution of FasR-670 SNPs.

Red clusters = Wild type allele (FasR-670A), Blue clusters = Mutant type allele (FasR-670G), Green clusters = Heterozygous (FasR-670AG) and Black clusters = Undetermined.

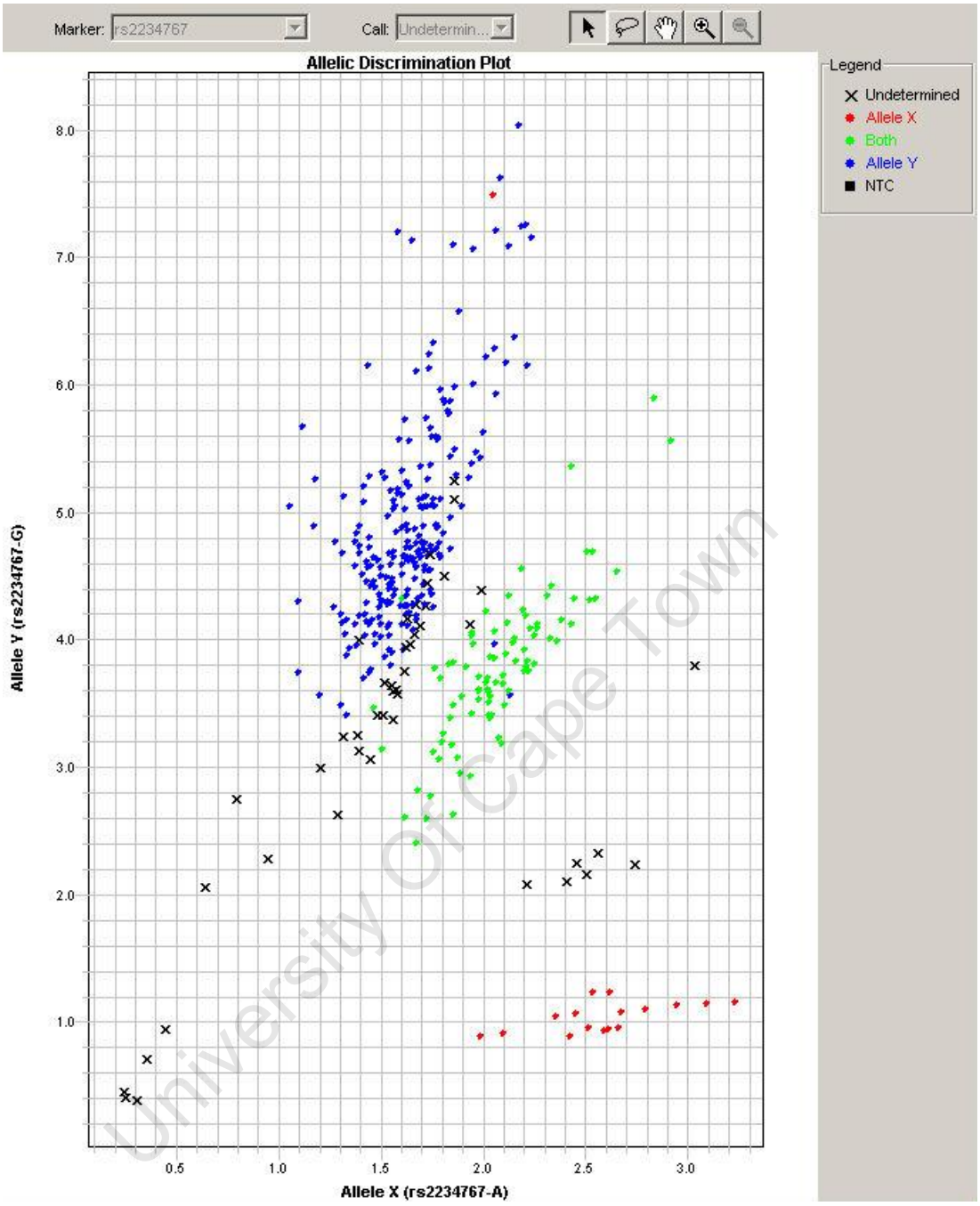

Fig. 2.2: One of the genotype clusters for TaqMan assay showing the distribution of FasR-1377 SNPs.

Red clusters = Wild type allele (FasR-1377G), Blue clusters = Mutant type allele (FasR-1377A), Green clusters = Heterozygous (FasR-1377GA) and Black clusters = Undetermined.

The genotyping was cross-checked by sequencing 4 samples for each polymorphism.

The sequencing results had a 100% reproducibility to the genotyping results.

Statistical Analysis

The genotype distributions were tested for deviation from Hardy-Weinberg equilibrium (HWE) in controls and also for linkage disequilibrium (LD) in cases and controls. HWE is carried out to see whether both allele and genotype frequencies in a population remain constant, that is, if they are in equilibrium from generation to generation. The main purpose for testing for deviation from HWE is to check for possible genotyping errors. Generally HWE is tested only in the controls and not in the cases. That is because the controls are randomly selected population which is a requirement of HWE. But cases are not random population and a group of handpicked individuals with a specific trait. For these analyses, D' and r^2 both were used to measure LD for different reasons. The P value described is measured in r^2 which is described as correlation coefficient. While the D' was used to see the distribution of the different loci and their co-relation with each other. Logistic regression was used to test for genotype, allelic and haplotype associations with cervix cancer status and the linear model was used to test for Genotypes against the Baseline characteristics (age, ethnicity and smoking status) and secondary outcomes such as HIV status, abnormal cytology (Pap smear) and high risk HPV according to Hybrid Capture II HPV Test in the control group. Logistic regression was the primary test used for all the analysis. Logistic regression was used since it adjusts for confounding factors. Other tests like Chi^2 do not adjust for confounding factors. We created a numerical variable that counted the number of minor alleles for the specific polymorphism and tested that in the logistic regression model. All analysis were adjusted for ethnicity and smoking. The distribution of different genotypes is known to differ among different ethnic groups. Smoking has been pointed in the literature as a true confounding factor of cervical cancer (reviewed in the Introduction and

Literature Review section). That led us to adjust for ethnicity and smoking to find the true modifiers of the disease.

FasR1377 and FasR-670 both are physically situated in a close proximity which might enable these two loci the power to influence each other. That led us to do the haplotype analysis with these two loci. We inferred haplotypes from the two polymorphisms in FasR, and tested their association with cervix cancer status and between ethnicities in the controls.

Statistical analyses were done with R, the freely available environment for graphics and statistics (http://www.r-project.org), and R packages dgc-genetics (http://www-gene.cimr.cam.ac.uk/clayton/software/) and haplo.stats (http://CRAN.R-project.org/package=haplo.stats). Broadly, haplo.stats uses the expectation maximisation (EM) algorithm, to calculate maximum likelihood estimates of probabilities, of haplotype pairs (one paternal, one maternal) for each subject, as described in Schaid et al. (Schaid et al., 2002) (assisted by the statistics collaborators, Dr. Lize van der Merwe and Dr. Ushma Galal (MRC, Cape Town)).

2.3 Results

The mean age for black cases was 43.8 yrs (SD 9.2) and for mixed-ancestry cases it was 45.9 yrs (SD 8.1). The mean age for black controls was 42.9 yrs (SD 9.0) and for mixed-ancestry controls it was 45.6 yrs (SD 8.2). No significant differences in age or HIV status were observed between cases and controls. The observed genotype frequencies of FasR-1377G/A, FasR-670A/G and FasL-844T/C in the controls did not deviate from Hardy-Weinberg equilibrium (FasR-1377: P = 0.20, FasR-670: P =

0.58, FasL-844: P = 0.12). The two FasR polymorphisms were in tight linkage disequilibrium (LD) (P < 0.05), except in the black controls (P = 0.078). Neither the allelic, nor the genotype frequencies showed a significant association (global P-values, 0.55, 0.12 and 0.77 for FasR-1377A, FasR-670A and FasL-844T respectively) with cervical cancer status (Table 2.1). The global P value is defined as the P value for the entire model considering at least 1 pair of all genotypes and haplotypes.

Table 2.1 - Counts (n), frequencies (%) and association statistics for genotypes and minor-alleles (FasR-1377G/A, FasR-670A/G, FasL-844T/C).

	Black		Mixed-ancestry		Genotype-cervical cancer association, adjusted for ethnicity		Genotype-ethnic association, in controls only	
	Cases	Controls	Cases	Controls				
Genotypes	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**P-value**	**OR (95% CI)**	**P-value**	**OR (95% CI)**
FASR–1377 G→A					0.550		0.004	
GG	74 (77)	75 (83)	186 (64)	196 (66)	-	1	-	1
AG	21 (22)	14 (16)	87 (30)	86 (29)	0.428	1.14 (0.83-1.58)	0.007	0.43 (0.22-0.77)
AA	1 (1)	1 (1)	17 (6)	13 (4)	0.391	1.37 (0.67-2.87)	0.130	0.20 (0.01-1.04)
A allele	23 (12)	16 (9)	121 (21)	112 (19)	0.275	1.15 (0.89-1.49)	0.003	0.43 (0.24-0.72)
FASR–670 A→G					0.120		0.001	
GG	67 (65)	54 (55)	147 (44)	136 (42)	-	1	-	1
AG	35 (34)	43 (43)	132 (40)	146 (45)	0.099	0.79 (0.59-1.05)	0.207	0.74 (0.46-1.18)
AA	1 (1)	2 (2)	53 (16)	39 (12)	0.492	1.17 (0.74-1.87)	0.006	0.13 (0.02-0.44)
A allele	37 (18)	47 (24)	238 (36)	224 (35)	0.778	0.97 (0.79-1.19)	0.003	0.57 (0.39-0.82)
FASL –844 T→C					0.770		<0.001	
TT	70 (68)	74 (74)	143 (44)	139 (44)	-	1	-	1
CT	31 (30)	23 (23)	144 (44)	136 (43)	0.515	1.1 (0.82-1.48)	<0.001	0.32 (0.18-0.53)
CC	2 (2)	3 (3)	40 (12)	40 (13)	0.929	0.98 (0.61-1.57)	0.001	0.14 (0.03-0.41)
C allele	35 (17)	29 (14)	224 (34)	216 (34)	0.797	1.03 (0.83-1.27)	<0.001	0.34 (0.22-0.51)

P-values and OR (95% confidence intervals) are for test of genotype or additive allelic association with cervix cancer risk, adjusted for ethnicity, and for genotype/additive association with ethnicity in controls. For genotypes, P-values next to genotype names are for joint model, others are for ORs of specific genotype compared to reference genotype, indicated with OR =1. ORs for alleles are the odds for an additional copy of that allele. ORs for genotype-ethnicity association (in controls only) are odds of genotype/allele in black compared to odds of genotype/allele in individuals of mixed-ancestry. Cases = Women with cancer of the cervix, Controls = Women without cancer of the cervix.

The haplotype analysis for FasR-1377/-670 also did not reveal any association with cervical cancer in either the black African (global P = 0.1130) (Black) or the mixed-ancestry (global P = 0.7043) population (Table 2.2).

Table 2.2 - Inferred frequencies, %, for FasR-1377 and FasR 670 haplotypes and odds ratios for risk of cervix cancer.

	Black			Mixed-ancestry		
Haplotype	**Cases**	**Controls**	**OR (95% CI)**	**Cases**	**Controls**	**OR (95% CI)**
GG	70	71	1	44	46	1
GA	18	20	0.8 (0.45-1.43)	36	35	1.08 (0.85-1.38)
AG	12	5	2.07 (0.87-4.93)	20	19	1.12 (0.83-1.52)
AA	0	4	-	0	0	-

Odds ratios (95% confidence intervals) are for testing FasR-1377 and FasR-670 haplotype association with risk of cervix cancer versus reference haplotype GG, stratified by ethnicity. Global P = 0.1130 (Black) and 0.7043 (Mixed ancestry).

The frequency of -1377A was 12% in black cases and 9% in the black controls, 21% in mixed-ancestry cases and 19% in mixed-ancestry controls. -670A was 18% in black cases and 24% in black controls, 36% in mixed-ancestry cases and 35% in mixed-ancestry controls. -844C was 17% in black cases and 14% in black controls, 34% in both mixed-ancestry cases and controls (Table 2.1).

The association of three SNPs with ethnicity was investigated only in the control group as the controls represent the larger South-African population. In the control group, all three SNPs showed significant allelic and genotype differences (global P-values, 0.004, 0.001, <0.001 for FasR-1377A, FasR-670A and FasL-844T respectively) between ethnic groups (Table 2.1). The FasR haplotypes (FasR-1377/-670) also showed a significant difference (global P-value <0.001) between the two ethnic groups among controls (Table 2.3).

Table 2.3 - Inferred frequencies, %, for FasR-1377 and FasR-670 haplotypes and odds ratios for comparing ethnicity in the control group.

Haplotype	Black	Mixed-ancestry	OR (95% CI)
GG	71	46	1
GA	20	35	0.45 (0.31-0.67)
AG	5	19	0.33 (0.19-0.58)
AA	4	0	-

Odds ratios (95% confidence intervals) are odds of being black compared to mixed-ancestry, for FasR-1377 and FasR 670 haplotypes versus reference haplotype GG, in the control group. Global P-value <0.001.

In the control group, both the GA and AG (FasR-1377 and FasR-670) haplotypes were more frequent in the mixed-ancestry group relative to black African group (AG: 19% compared to 5%; GA: 35% compared to 20%), while the GG haplotype was more common in blacks (71%) as compared to the mixed-ancestry group (46%).

No significant association was found between any of the secondary outcomes (HIV infection, abnormal cytology (Pap smear) and hybrid capture high risk HPV) and any of the three SNPs in the control group, adjusted for ethnicity (results not shown here).

2.4 Discussion

Polymorphisms in the Fas or FasL genes that impair the apoptotic signals have been associated with susceptibility to a number of different cancers (Davidson et al., 1998;Lee et al., 1999;Peters et al., 1999;Sun et al., 2004;Takahashi et al., 1994;Zhang et al., 2005) including cervical cancer (Dybikowska et al.,

2004;Engelmark et al., 2004;Ivansson et al., 2007a;Lai et al., 2003;Lai et al., 2005;Sun et al., 2005). We are the first to study the frequency of the FasR-1377G/A, FasR-670A/G and FasL844T/C polymorphisms in an indigenous black African as well as in a mixed-ancestry population and found no association with cancer of the cervix. The tendencies of the odds ratios (ORs) (FasR-1377A/A 1.37, FasR-670A/A 1.17) point towards an increasing susceptible effect and a need for a larger study. Although the result was not statistically significant, there were more cases with FasR-1377A variant compared to controls in both black African (12% vs. 9%) and mixed-ancestry (21% vs. 19%) women. Our results agree with the lack of association of Fas SNPs with cervical cancer shown by three other reports (Dybikowska et al., 2004;Engelmark et al., 2004;Ivansson et al., 2007a). The differing results between studies might be due to one or several factors, including the difference in ethnic origin of populations studied and the difference in sample size. The presence of another causative mutation in tight LD with any of these three polymorphisms can also not be ruled out.

We observed statistically significant allele frequency differences for all the three Fas SNPs between ethnic groups in the control women. For FasR-1377G/A, the A allele frequency was 9% in the black Africans and 19% in women of mixed-ancestry, which were both markedly lower than the frequency in other populations, such as the 43% and 31% in Taiwanese and Chinese populations, respectively (Lai et al., 2003;Sun et al., 2005). The FasR-670A allele frequency was 24% in black Africans and 35% in women of mixed-ancestry. This is again lower than the average of 53% for populations from Europe, Australasia and Asia (Dybikowska et al., 2004;Huang et al., 1997;Lai et al., 2003;Niino et al., 2002;Seo et al., 2002). The frequency of the

FasL-844C alleles was 14% in black women and 34% in women of mixed-ancestry, which are very low compared to the 69% and 71% in the Chinese and Swedish populations respectively (Ivansson et al., 2007a;Sun et al., 2005). The unusually low frequency of the Fas and FasL alleles in the African population could contribute to a lower power to detect an association in the present study.

The FasR-1377G/A and FasR-670A/G SNPs are in tight LD in our population. Both the AG and GA haplotypes were significantly more frequent in women of mixed-ancestry compared to GG, the most common haplotype, which was observed at a higher frequency in black women (Table 2.3).

2.5 Conclusion

Our study did not show a significant association of the FasR-1377G/A, FasR-670A/G and FasL-844T/C polymorphisms with cervical cancer. A statistically significant difference was found in the allele and genotypes frequencies of the three SNPs between women of black African and mixed-ancestry in the control population.

HSV-2 is a well known STD and a risk factor cervical cancer. Very little work has been done on host genetic susceptibility factors contributing to HSV-2 infection. We wanted to find out if the cell death pathway genes, Fas and FasL anyway influence the susceptibility to HSV-2 infection in our population.

CHAPTER 3

A FAS GENE POLYMORPHISM INFLUENCES HERPES SIMPLEX VIRUS TYPE 2 INFECTION IN SOUTH AFRICAN WOMEN

3.1 Introduction

Herpes simplex virus type 2 (HSV-2) is a common sexually transmitted virus causing genital infections which persist as life-long latent infection in sensory neurons. The prevalence of HSV-2 infection is high in the developing world, especially in sub-Saharan Africa with higher incidence in women compared to men (Ivansson et al., 2007b). Immune surveillance by T cells plays an important role in regulating virus infection in human. Activation induced cell death (AICD), regulated by the Fas-FasL pathway is a crucial mechanism to eliminate virus-infected cells by inducing apoptosis or programmed cell death (Zhang et al., 2004). Therefore, two of the best non-HLA genes which are good candidates to investigate if they play a role in susceptibility to HSV-2 infection are Fas/CD95 and Fas ligand.

Gene polymorphisms that impact on the apoptotic process will also potentially interfere with the processes associated with viral spread and clearance. Fas ligand binds to the Fas receptor and activates pro-caspase 8 which forms caspase 8 that directs the cells to apoptosis. The signal emanated through transcription factor binding sites of stimulatory protein 1 (SP1) and signal transducer and activator of transcription 1 (STAT1) is associated with transcriptional activation and expression

of the Fas gene. Three functional polymorphisms in this pathway, namely FasR-1377G/A, FasR-670A/G and FasL-844T/C have been well studied which might alter the cellular apoptosis by dysregulation of Fas-FasL pathway and have also been associated with different diseases (Lai et al., 2003;Sibley et al., 2003;Wu et al., 2003). FasR-670G/G has been associated with protective effect to HIV-related lipodystrophy,(Lai et al., 2003) FasR-670A/A conferred a susceptible effect to adult T cell leukemia (human T lymphotropic virus type 1-infection) (Lai et al., 2003) and cervical carcinogenesis (Lai et al., 2003). FasR-1377A/A has been associated with susceptibility to squamous cell carcinoma of the head and neck.(Lai et al., 2003) FasL-844T/C has not been associated with any viral infections.

The aim of this study therefore was to investigate role of the three single nucleotide polymorphisms from non-HLA genes on individual susceptibility to HSV-2 infection.

3.2 Herpes Simplex Virus Type-2

HSV-2 belongs to the *Alphaherpesviridae* group. It is a large, linear, double-stranded DNA virus (Fig. 3.1) (Novak and Peng, 2005).

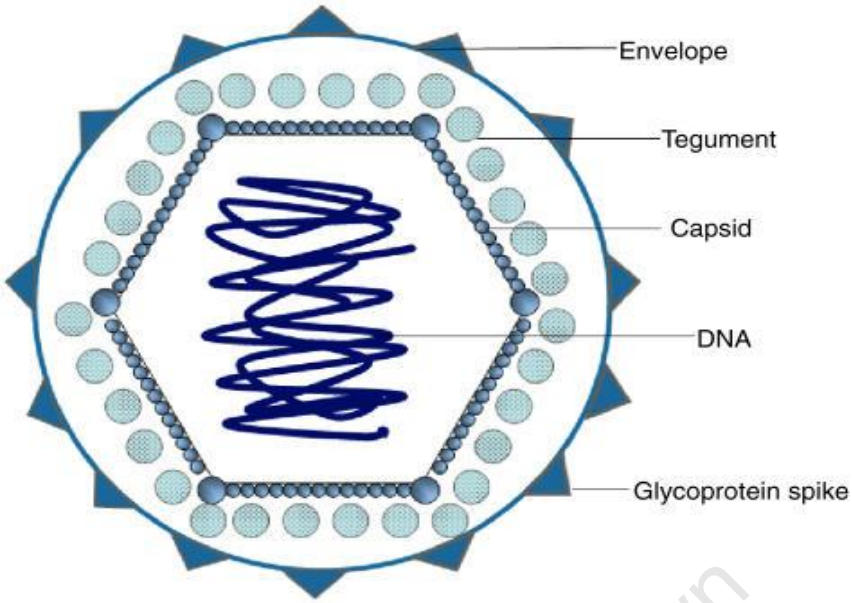

Fig. 3.1: A schematic diagram showing the structural conformation of HSV-2 (Novak and Peng, 2005).

The genome is packaged into an icosahedral capsid (Shukla and Spear, 2001). This capsid is surrounded by a layer of protein called tegument. The tegument is covered by a host-cell derived lipid bilayer containing viral different glycoproteins and spikes. The glycoproteins are essential for viral infectivity (Akhtar and Shukla, 2009;Duerst and Morrison, 2003).

3.2.1 Herpes Simplex Virus type-2 infection

HSV-2 is a common sexually transmitted virus that causes genital infections. HSV-2 is the major causative agent for genital herpes infection (Bukowski and Welsh, 1986) which persists as life-long latent infection in sensory neurons. The virus remains latent in neurons after initial infection (Shukla and Spear, 2001). During the latency period virus can still spread in infected hosts by asymptomatic shedding of the virions to other humans. The virus can be reactivated by a variety of environmental triggers, such as emotional or physical stress (Whitley and Roizman, 2001). The

reactivation of the virus leads to replication of the virus in epithelial cells and a lifetime of intermittent mucocutaneous lesions (Whitley and Roizman, 2001). Genital herpes infection is also the primary cause of genital ulcer disease (GUD) across the world (World Health Organization, 2001), mainly caused by HSV-2. HSV-2 can also cause life-threatening diseases in immunocompromised individuals, newborns from infected mothers resulting in fatal encephalitis and in patients with HIV (Shukla and Spear, 2001;Whitley and Roizman, 2001). One in 3,500 births in the US is affected by HSV-2 infections (Schomogyi et al., 1998). Transmission of HSV-2 from one host to another requires sexual intercourse.

Only a handful of countries worldwide have a population-based national estimate of HSV-2 to keep track of the spread of HSV-2 infection. A survey in the United States indicated an increase in age-adjusted HSV-2 prevalence from 16.4% to 21.7% from 1976 to 1994 (Fleming et al., 1997). An European cross-sectional survey on age-standardised HSV-2 seroprevalence conducted between 1989 to 2000 observed a range of 4% in England and Wales to 24% in Bulgaria (Pebody et al., 2004). The prevalence of HSV-2 infection is much higher in the developing world, especially in sub-Saharan Africa with a higher incidence in women compared to men. Age-adjusted HSV-2 seroprevalence in adults ranges from 30% to 80% in women and 10% to 50% in men (Weiss, 2004).

Research on the development of a vaccine for protection against HSV-2 infection is ongoing (Koelle and Corey, 2008). HSV-2 is known to have different adaptations to their human hosts pointing to an evolutionary ancient relationship. This adaptation capacity of HSV-2 has repeatedly hampered the efforts to develop an effective

vaccine. It has been pointed that regular use of antivirals such as, acyclovir and valacyclovir can reduce HSV-2 shedding by 60-80% and can cut HSV-2 transmission risk to almost half (Koelle and Corey, 2008).

3.2.2 Herpes Simplex Virus type-2 entry into host cell and its life cycle

HSV-2 entry into host cells is possibly the most crucial step in viral pathogenesis. HSV-2 glycoproteins play an important role in viral entry into host cells. Five viral glycoproteins have been identified in viral entry process, namely gB, gC, gD, gH and gL (Campadelli-Fiume et al., 2007;Spear, 2004). Other than gC all the glycoproteins are essential for viral entry into host cells. Though gC is not essential for viral entry, its absence decreases the viral binding to cell-surfaces (Shukla and Spear, 2001). The glycoproteins attach to the cell-surface and help the virus to enter into the cell. HSV-2 entry into host cell and its replication is shown in the following diagram (Fig. 3.2).

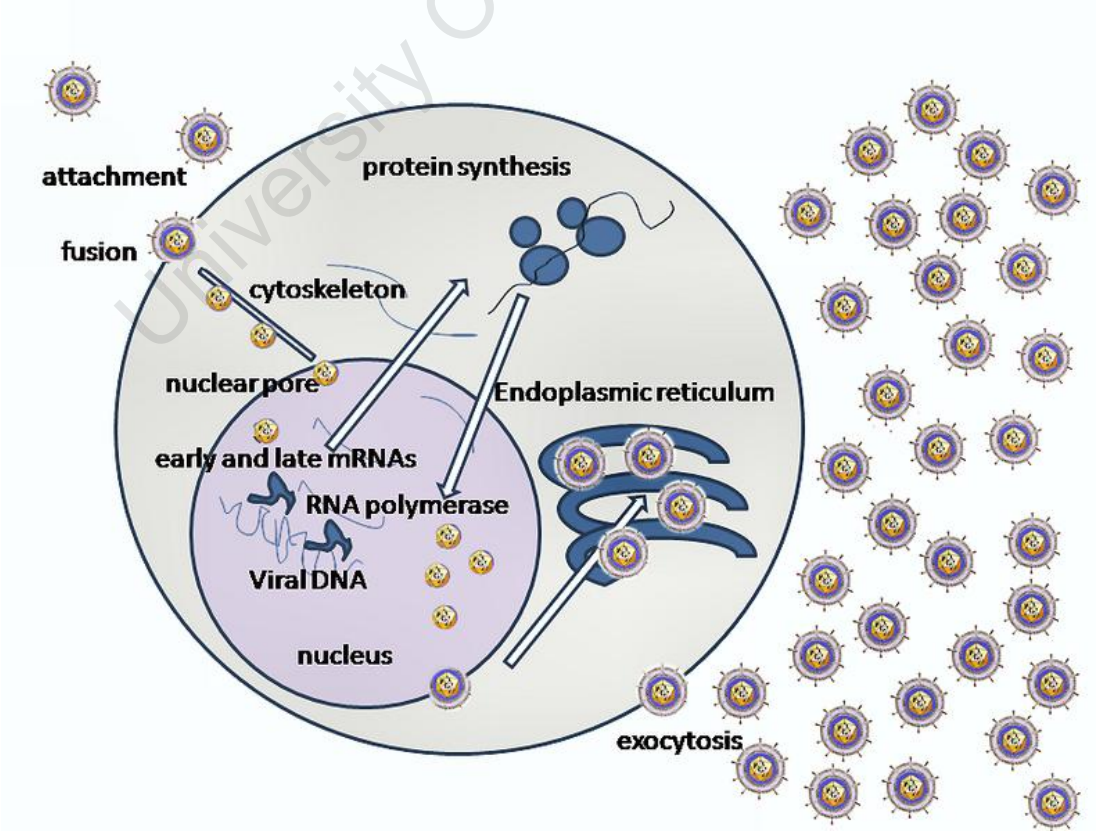

Fig. 3.2: HSV-2 entry into host cell and its replication (Graham Colm, 2007)

The virus enters inside the host cells by two major modes of entry (Akhtar and Shukla, 2009). I) HSV-2 virions can enter into cells via a pH-independent fusion of viral membrane with the plasma membrane. II) The virus can also enter into the cell via an endocytic pathway. For both the pathways HSV-2 particles initially associate with filopodia-like membrane protrusions via heparan sulphate proteoglycans (HSPGs) (Akhtar and Shukla, 2009). After attachment, the virus penetrates through the cell-surface to the cytoplasm. As the virus fuses with a cellular membrane the viral nucleocapsid and tegument proteins get released into the host cytoplasm (Akhtar and Shukla, 2009). In post-entry steps, HSV-2 nucleocapsids dissociate with tegument proteins and bind to dynein motor (Sodeik et al., 1997). Most of the tegument proteins participate in activation and modulation of viral gene expression and shut-down of host protein synthesis. Some tegument proteins may also participate in transport of the nucleocapsids toward the nuclear membrane for uncoating and release of viral DNA into the host nucleus (Akhtar and Shukla, 2009). Inside the host nucleus, the virus replicates and assemble to form a new virus (Akhtar and Shukla, 2009). After replication the virus spreads to another cell and the infection grows.

3.3 Immune response to Herpes Simplex Virus type-2 infection

3.3.1 Innate immune response to Herpes Simplex Virus type-2

Innate immune mechanisms are rapid response to an infection and a relatively undiversified reponse. Innate immune responses to virus infection have three phases: complement protein and natural-antibody mediated responses, early induced responses such as interferons produced by infected epithelia and resident dendritic

cells (DC), neutrophils, macrophages and NK cells (Duerst and Morrison, 2003). When an HSV-2 virus attempts to infect the female genital mucosa it first encounters mechanical defences that provides a constant barrier to infection. Mucus, Bacterial flora and glycocalyx can act as mechanical defense. Genital tract secretions such as complement and IgM can also prevent HSV-2 entry into epithelial cells (Duerst and Morrison, 2003). If some of the HSV-2 virus breach this defense and replicate in epithelial cells, this triggers further activation of complement and stimulates the production of chemokines and IFNαβ. These substances alert the DCs and macrophages for the presence of HSV-2. The immature DCs carry the HSV-2 antigens to regional lymph nodes and stimulate T cell activation (Zhou X. et al., 2003). This helps to alert the systemic adaptive immune response (Duerst and Morrison, 2003). After DCs exit the mucosa, cells of the late-induced innate response enter. An influx of neutrophils, monocytes and NK cells flood the infected region following the gradient of chemokines radiating from the site of infection. Once in the infected site, they phagocytose and destroy virus particles and infected cells by producing antiviral substances (Duerst and Morrison, 2003). These substances also help to activate the adaptive immune response against HSV-2.

3.3.2 Adaptive immune response to Herpes Simplex Virus type-2

Classical adaptive immune responses are mediated by T and B lymphocytes with enhanced memory and exclusive antigen specificity. DCs are the most potent APCs for stimulating T lymphocytes. The migrations of the DCs to regional lymph nodes mature them and help to display viral peptides on MHC molecules. DCs also secrete cytokines at the same time. The cytokines direct differentiation of the Th0 cells to

Th1 or Th2 cells. Macrophages and NK cells also produce cytokines that direct Th1 differentiation, including IL12, IL-18 and IFNγ. T cells especially CD4+ Th1 cells and their production of IFNγ are essential for clearing HSV-2 infection (Manickan and Rouse, 1995;Milligan et al., 1998).

3.4 Herpes Simplex Virus type-2 immune evasions

Immune surveillance by T cells plays an important role in regulating virus infection in humans. Activation induced cell death (AICD), regulated by the Fas-FasL pathway is a crucial mechanism to eliminate virus-infected cells by inducing apoptosis or programmed cell death (Zhang et al., 2004).

In order for HSV-2 to evade the immune system, it has to overcome the apoptosis of the cell which is triggered by the activation of Fas-FasL pathway. HSV evades the immune system in two phases, an early anti-apoptotic phase and a late pro-apoptotic phase (Fig 3.3) (Novak and Peng, 2005).

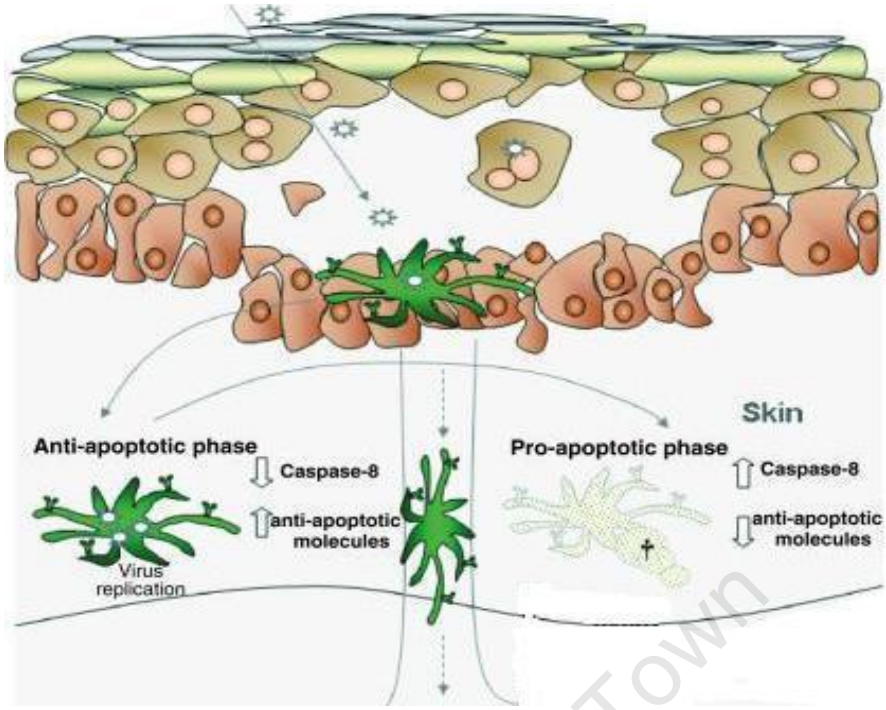

Fig. 3.3: Interaction between HSV and caspase 8 (Novak and Peng, 2005)

In the early anti-apoptotic phase, HSV produces glycoprotein D which activates cellular protein NF-κB. NF-κB upon activation enters the nucleus, binds to the DNA and reduces the expression of caspase 8 and up-regulates intracellular anti-apoptotic molecules which protect the virus-infected cell from Fas-FasL pathway mediated apoptosis (Medici et al., 2003). HSV-2 is able to down-regulate the expression of Fas L which presents as one mechanism to reduce apoptosis (Sieg et al., 1996). By these above processes the virus ensures the survival of the host cell for a sufficient time for the virus to replicate. In the second phase, HSV activates Fas-FasL mediated apoptosis of immature dendritic cells by down regulation of anti-apoptotic molecules and up-regulation of caspase 8. Fas or porforin- mediated cytolysis is essential for clearance of HSV-2 from genital epithelium (Dobbs et al., 2005). The elimination of the dendritic cells helps the virus to further avoid the immune system and the infection spreads.

3.5 Host genetic risk factors to Herpes Simplex Virus type-2 infection

There is a report on the association of HLA alleles with HSV-2 infection in a Caucasian American cohort. HLA-B27 and -Cw2 showed a protective effect to symptomatic HSV-2 infection. On the other hand, HLA-Cw4 showed a susceptible effect to HSV-2 infection when compared with non-infected individuals (Lekstrom-Himes et al., 1999). To the best of our knowledge, no previous studies have investigated the roles of Fas/FasL polymorphisms in susceptibility to HSV-2 infection.

3.6 Materials and Methods

Study Participants

Participants were 424 controls (101 black African and 323 women of mixed-ancestry) without cancer of the cervix. All participants were unrelated subjects. The study was approved by the University of Cape Town Research Ethics Committee (REC REF: 075/2009). This is a secondary analysis focusing on risk of HSV-2 infection in the women who did not have cervical cancer.

HSV-2 Detection

The HSV-2 status of 407 of these 424 women was assessed. HSV-2 was detected by HerpeSelect® 2 IgG enzyme-linked immunosorbent serologic assay (ELISA) (Focus

Diagnostics, Inc., CA, USA) screening of the sera aliquots according to manufacturer's instructions. Antibodies to HSV-2 are markers of infection. There was no information available on which women were asymptomic or alternatively shedding HSV-2. This test has been validated by other researchers as a method of testing for HSV-2 antibodies in Africans (Shapiro et al., 2003). The index cutoff value for our test was 0.90 which was calculated as an average of four optical density (OD) calibrators and was in line with the company manuals supplied with the kit. There were 84.2% high positives (3.5 OD or greater) and 15.3% were low positives (below 3.5 OD, but greater than 1.1 OD). Values between 0.9 OD and 1.1 OD were considered as indeterminate. There were very few indeterminate values (0.5%) which will not affect the outcome of our results (this work was carried out by the NHLS Medical Virology Division, Groote Schuur Hospital, Cape Town, Republic of South Africa).

Extraction of Genomic DNA and Genotyping

Reactions were conducted in 5 µl volumes and contained 10 ng DNA, 1.75 µl PCR MasterMix (Applied Biosystems), 0.087 µl TaqMan assay mix (FasR-1377 (rs2234767, assay id, C_12123966_10), FasR-670 (rs1800682, assay id, C_9578811_10), FasL844 (rs763110, custom-designed)) (Applied Biosystems) and 1.16 µl distilled water. Cycling conditions on the ABI prism 7900 HT (Applied Biosystems) were 2 minutes at 50˚C, 10 minutes at 95˚C, followed by 40 cycles of 15 seconds at 92˚C and one minute at 60˚C. End point fluorescence was measured immediately after cycling. Alleles were assigned using SDS 2.0 software (Applied Biosystems) (Zoodsma et al., 2005) (this work was carried out at Uppsala University,

Sweden). The genotyping was cross-checked by sequencing 4 samples for each polymorphism.

Statistical Analysis

The genotype distributions were tested for deviation from Hardy-Weinberg equilibrium (HWE) and also for linkage disequilibrium (LD). HWE is carried out to see whether both allele and genotype frequencies in a population remain constant, that is, if they are in equilibrium from generation to generation. Logistic regression was used to test for genotype, allelic and haplotype associations with HSV-2 positivity status as well as baseline characteristics (age, ethnicity and smoking status) and secondary outcomes such as HIV status, abnormal cytology (Pap smear) and high risk HPV according to Hybrid Capture II HPV Test. We created a numerical variable that counted the number of minor alleles for the specific polymorphism and tested that in the logistic regression model.

We inferred haplotypes from the two polymorphisms in FasR, and tested their association with HSV-2 infection status and between ethnicities in the group under investigation.

Statistical analyses were done with R, the freely available environment for graphics and statistics (http://www.r-project.org), and R packages dgc-genetics (http://www-gene.cimr.cam.ac.uk/clayton/software/) and haplo.stats (http://CRAN.R-project.org/package=haplo.stats). Broadly, haplo.stats uses the expectation maximisation (EM) algorithm, to calculate maximum likelihood estimates of

probabilities, of haplotype pairs (one paternal, one maternal) for each subject, as described in Schaid et al. (Schaid et al., 2002).

Logistic regression enabled us to adjust all tests for the known confounder, ethnicity (genotype and allele frequencies differed significantly between the ethnic groups), by including it as a covariate in each model. We did not adjust our results for multiple testing. We felt that a Bonferroni adjustment, multiplying all p-values with 3, would be very conservative when taking into account that the SNPs were in tight LD (results not shown) (assisted by the statistics collaborators, Dr. Lize van der Merwe and Dr. Ushma Galal (MRC, Cape Town)).

3.7 Results

The mean age for the HSV-2 infected Black women was 42.8 (SD 8.8) and for the HSV-2 infected women of mixed-ancestry it was 46.6 (SD 7.6). The mean age for the HSV-2 non-infected Black women was 42.8 (SD 11.1) and for the HSV-2 non-infected women of mixed-ancestry it was 43.9 (SD 8.8). The mean age among the women whether HSV-2 infected and non-infected were not significantly different. Among black women, 89% were positive for HSV-2 infection and among mixed-ancestry women 58% were positive for HSV-2 infection. A statistically significant association (p-value = 0.029) was found between the FasR-1377G/A SNP and HSV-2 infection, after adjusting for ethnicity. The FasR-1377A allele were associated with a highly significant decreased risk (p-value = 0.008, OR = 0.58 (95% CI: 0.38-0.87)) of HSV-2 infection, after adjusting for ethnicity, with a frequency of 41.5% in HSV-2 non-infected women and 22.6% in infected women (Table 3.1).

Table 3.1: Counts (n) and percentage (%) distributions for genotypes (FasR-1377G/A, FasR-670A/G, FasL-844T/C), and minor alleles. P-values and odds ratios for association with HSV-2 status, adjusted for ethnicity.

	Black		Mixed-ancestry		SNP-HSV-2 association, ethnicity-adjusted	
	HSV-2 infected	HSV-2 non-infected	HSV-2 infected	HSV-2 non-infected		
n	**88**	**11**	**177**	**131**		
	n (%)	**n (%)**	**n (%)**	**n (%)**	**P-value**	**OR (95% CI)**
FASR–1377 G→A					0.029	
GG	70 (85)	5 (71)	117 (73)	73 (60)		1
AG	12 (15)	1 (14)	38 (24)	42 (34)	0.033	0.57 (0.35-0.96)
AA	0 (0)	1 (14)	5 (3)	7 (6)	0.069	0.33 (0.09-1.06)
A allele	12 (7)	3 (21)	48 (15)	56 (23)	0.008	0.58 (0.38-0.87)
FASR–670 A→G					0.486	
GG	46 (53)	7 (64)	76 (43)	56 (43)		1
AG	38 (44)	4 (36)	82 (47)	55 (42)	0.576	1.14 (0.72-1.8)
AA	2 (2)	0 (0)	18 (10)	19 (15)	0.406	0.74 (0.36-1.52)
A allele	42 (24)	4 (18)	118 (34)	93 (36)	0.724	0.94 (0.68-1.3)
FASL–844 T→C					0.829	
TT	2 (2)	0 (0)	21 (12)	18 (14)		1
CT	22 (25)	1 (9)	74 (43)	54 (42)	0.612	1.13 (0.71-1.81)
CC	63 (72)	10 (91)	76 (44)	57 (44)	0.869	0.94 (0.47-1.92)
C allele	26 (15)	1 (5)	116 (34)	90 (35)	0.920	1.02 (0.74-1.40)

P-values and odds ratios are for test of genotype/additive association with herpes risk, adjusted for ethnicity. P-values next to SNP names are for joint model; others are for odds ratios of specific genotype compared to reference genotype (indicated with OR=1). ORs for alleles are the odds for each additional copy of that allele.

The significance of this result remains even after a Bonferroni adjustment of multiplication with 3. No statistically significant association was found between FasR-670A/G or FasL-844T/C polymorphisms and risk of HSV-2. A highly significant association (Global p-value = 0.0001) was found between HSV-2 and the FasR-1377/FasR-670 AG haplotype (Table 3.2).

Table 3.2: Inferred frequencies (%), and association statistics for testing FasR-1377 and FasR-670 haplotype association with risk of HSV-2, stratified by ethnicity. Global p-value = 0.0001.

Haplotype	HSV-2 infected	HSV-2 non-infected	OR (95% CI)
GG	57	43	1
GA	31	34	0.66 (0.47-0.93)
AG	12	23	0.42 (0.28-0.64)
AA	-	-	-

Specifically, the FasR-1377/-670 AG haplotype showed a decreased risk (OR = 0.42 (95% CI: 0.28-0.64)) of HSV-2 infection with a frequency of 12% in HSV-2 infected women and 23% in HSV-2 non-infected women compared to the GG haplotype, with a frequency of 57% for HSV-2 infected compared to 43% for HSV-2 non-infected women (Table 3.2). The FasR-1377/-670 GA haplotype also showed a decreased risk (OR = 0.66 (95% CI: 0.47-0.93)) of HSV-2 infection with a frequency of 31% in HSV-2 infected women and 34% in HSV-2 non-infected women compared to the GG haplotype (Table 3.2).

3.8 Discussion

We assessed the association between HSV-2 status and genotype, allelic (additive) and haplotype respectively, for three SNPs in a study group consisting of two ethnic groups. We did not test the ethnic groups separately, because we know, from the original study (Hogrefe et al., 2002), that the allele and genotype frequency

distributions are not the same for the ethnic groups. Therefore, we adjusted for ethnicity by including it as a covariate in the logistic regression models.

Our analysis showed that FasR-1377A allele was associated with reduced risk of infection with HSV-2, after adjusting for ethnic origin. The association was further confirmed in the FasR-1377A/G genotype. The observation of an association of the AG haplotype of FasR-1377/-670 with reduced HSV-2 infection could mainly be due to the FasR-1377A allele since the FasR-670A/G polymorphism did not show significant difference between infected and uninfected when analyzed separately.

The life cycle of HSV is extremely complex. The first step is viral entry where multiple HSV and cellular proteins play a role (Heldwein and Krummenacher, 2008). At different times of the HSV-2 life cycle apoptosis is promoted to allow virus to spread to other cells or apoptosis is prevented during periods of latency. Therefore, HSV is able to affect the apoptotic pathways in many ways and these are also dependent on the phase of the life cycle and which cells are infected (Carter, 2008). However, these apoptotic events are associated with events post HSV infection and not with events that could be associated with preventing HSV infection. Our study has shown a possible link with FasR-1377A allele and the reduction of HSV-2 infection. This allele is associated with decreased Fas expression which would imply less apoptosis. The possible mechanism of this protection needs further investigation.

3.9 Conclusion

In conclusion, our study reports on a possible role for FasR-1377A/G SNP in HSV-2 infection which is a novel result. This information adds on the growing literature on the host genetic susceptibility factors to HSV infection. However, the main limitation of this study is that it was done as a secondary analysis. The results should be interpreted cautiously as the subjects in this study were not selected primarily for the study of HSV-2 infection; however, these findings open the possibility of new studies focusing on other non-HLA genetic factors in HSV-2 infection. Further studies on well defined and large cohorts are needed to further confirm our findings and to elucidate the mechanisms involved.

CHAPTER 4

CASP8 PROMOTER POLYMORPHISM IS ASSOCIATED WITH HIGH-RISK HPV TYPES AND ABNORMAL CYTOLOGY BUT NOT WITH CERVICAL CANCER IN SOUTH AFRICAN WOMEN

4.1 Introduction

Cervical cancer is a multistep process that develops slowly over several years due to persistent infection of the epithelial cells with human papillomavirus (HPV). HPV infection may lead to atypical squamous cells of undetermined significance (ASCUS) or squamous intraepithelial lesions (SIL) (Rohan et al., 2003). 90% of ASCUS regresses to normal cytology. Approximately 30.8% of the ASCUS positive individuals are HPV-infected according to Digene Hybrid Capture II test (Allan et al., 2006) while the rest could be due to other stresses by different environmental factors. Approximately 63.2% of the LSIL and 83% of the HSIL positive individuals are HPV-infected according to Digene Hybrid Capture II test (Allan et al., 2006). LSILs are precancerous and are very early precursor stages of cervical cancer with very few cases progressing to cancer (Snijders et al., 2006). On the other hand, most HSILs progress to invasive cervical cancer (ICC) if not treated on time.

The human immune system eliminates HPV-infected cells and the pre-cancerous malignant cells by apoptosis (Garnett and Duerksen-Hughes, 2006). The spread of cancer depends on successfully overcoming the apoptotic mechanism of the immune

system. Any defect in this pathway leads to inefficiency of the apoptotic mechanism which helps in cancer progression (Cotter, 2009). Caspase-8 is one of the most important proteins in this pathway that generates apoptotic signals. Functional polymorphism at the CASP8 gene that encodes Caspase-8 might lead to altered apoptotic signals affecting the spread of the pre-cancerous lesions. *CASP8* -652 6N ins/del polymorphism has been studied in different populations as described in the literature review. But no investigation has been done with this polymorphism in African population. This polymorphism has also been shown to influence the susceptibility to cervical cancer in one of the studies (Sun et al., 2007). This variant has a direct functional effect on risk of tumour progression as it decreases *CASP8* transcription and hence reduction in caspase-8 expression (Sun et al., 2007). Biochemical analyses have also shown that T lymphocytes with the deletion variant display reduced caspase-8 activity and reduced AICD when stimulated with cancer cell antigens (Sun et al., 2007).

4.2 Materials and Methods

Participants

DNA from a total of 1854 subjects comprising 447 women with invasive cervical cancer (106 black African and 341 women of mixed-ancestry) and 1407 controls (314 black African and 1093 women of mixed-ancestry) without cancer of the cervix was studied. There were 264 (59%) urban cases and 183 (41%) rural cases compared to 715 (53%) urban controls and 632 (47%) rural controls (residency status was not known for 60 controls)..

The mean age for black cases was 43.8 yrs (SD 9.2) and for mixed-ancestry cases it was 45.9 yrs (SD 8.1). The mean age for black controls was 42.3 yrs (SD 9.1) and for mixed-ancestry controls was 44.3 yrs (SD 8.4). The HIV infection was 5% for the cases and 4.7% for the controls. No significant differences in age or HIV status were observed between cases and controls (data not shown here). Among 1230 controls for which pap smear results were available, 180 (15%) were abnormal (87 (7%) with SIL (LSIL and HSIL), 43 (3%) with HSIL and 93 (8%) ASCUS) and 1050 (85%) were normal. Smoking activity differed significantly between the cases and the controls (P = 0.001, OR (95% CI) = 1.60 (1.23-2.07), adjusted for ethnicity) (data not shown here). Subsequently, all the analyses were adjusted for the smoking and ethnicity.

Papanicolaou test

Papanicolaou test (Pap smear) was conducted on endocervical scrapings taken from the control women to test for cervical cytology as previously described (Shapiro et al., 2003). For this study, samples displaying ASCUS (atypical squamous cells of undetermined significance), LSIL or HSIL were considered as abnormal cytology.

High-risk HPV type detection

Endocervical scrapings from control women were assayed for HPV infection using the Hybrid Capture II HPV Test for the detection of high risk HPV types 16/18/31/33/35/39/45/51/52/56/58/59/68, and classified positive according to the manufacturer's instructions (Digene Corporation, Gaithersburg, MD, USA) as described earlier (Shapiro et al., 2003). Of controls, 15% (180/1230) for which a

Hybrid Capture test was done were positive and 85% (1050) were negative. Each female was represented by a single cytological specimen.

Extraction of genomic DNA

The genomic DNA was extracted using TotalNucleicAcid Extraction kit for MagNA Pure Compact nucleic acid extractor (Roche Diagnostics, Germany).

Determination of CASP8 -652 6N ins/del polymorphism

The *CASP8* -652 6N ins/del polymorphism (rs3834129:–/AGTAAG; NM_001228.4) was determined using a polymerase chain reaction (PCR) – restriction fragment length polymorphism (RFLP) method followed by agarose gel electrophoresis as described by Sun T et. al. (Sun et al., 2007). PCR reactions were carried out for each sample using a forward primer and a reverse primer. 130ng of genomic DNA was amplified in a 10-µl reaction mixture containing 10picomoles of each *CASP8* -652 6N ins/del primers: F, 5'CTGCATGCCAGGAGCTAAGT3' and R, 5'GCCATAGTAATTCTTGCTCTGC3' (Sun et al., 2007) and 5-µl 2x ImmoMix^{TM} (Bioline). PCR cycle reactions were performed on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) beginning with a denaturing step at 94°C for 2 min followed by 30 cycles of denaturing at 94°C for 20 s, annealing at 53°C for 10 s and extension at 72°C for 15 s followed by a final extension at 72°C for 5 min. The cases and controls were generally not mixed in plates except for a few occasions. At times when cases and controls were mixed on plates, the statuses of the samples were unknown and the genotype calling was blind to case-control status. A DNA free negative sample, known positive sample for wild type homozygous and known positive sample for mutant homozygous were controlled for. Every genotyping plate

had a positive control sample included and it was always picked up as positive giving us assurance of the obtained genotypes were the correct ones.

The primers generated PCR products of either 177bps (*CASP8* -652 6N ins) or 171bps (*CASP8* -652 6N del). The amplified PCR products were digested by the *Bfa*I restriction enzyme (New England Biolabs, MA, USA) using New England Buffer 4 (10x) for overnight.

The digested PCR products were analysed on 1.5% agarose gel stained with ethidium bromide using a 25bp DNA Step Ladder (Promega Corporation, Madison, USA). The samples with *CASP8* -652 6N ins variant produced two fragments of 31 base pairs (bps) and 146bps. The samples with *CASP8* -652 6N del allele produced one fragment of 171bps. Heterozygous samples (*CASP8* -652 6N ins/del) produced all the three fragments of 31bps, 146bps and 171bps. The fragment of 31bps was not detected in our gel analysis due to its low intensity (Fig. 4.1).

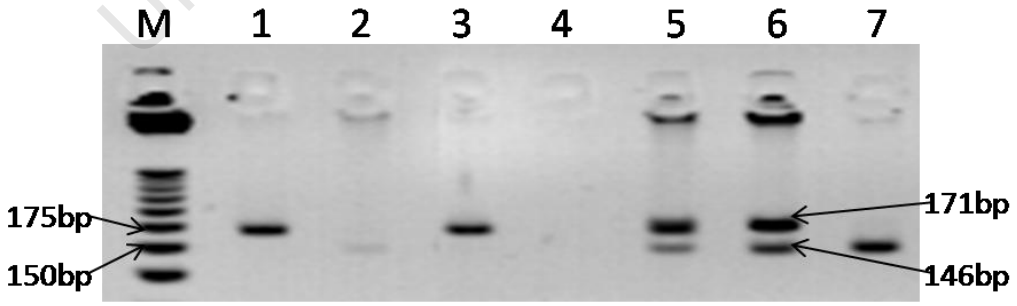

Figure 4.1 - Analysis of the *CASP8* -652 6N ins/del polymorphism on agarose gel.

M = DNA ladder, samples 1 and 3 = *CASP8* -652 6N ins/ins (wild type homozygous), samples 5 and 6 = *CASP8* -652 6N ins/del (heterozygous), sample 7 = *CASP8* -652 6N del/del (mutant type homozygous), samples 2 and 4 = PCR did not work.

DNA sequencing was carried out in a random manner for 40 samples to cross-check the genotyping results. The same forward primer (previously used for genotyping of CASP8 -652 6N ins/del) and a new reverse primer (5'CCTCTTCAATGCTTCCTTGAG3') was used for sequencing both DNA strands (forward and reverse). The DNA sequencing was done using a BigDye Teminator V3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA) following the manufacturers protocol. The reproducibility of the sequencing results were 100% to the previous genotyping results.

Statistical Analysis

The genotype distributions were tested for Hardy-Weinberg equilibrium in cases and controls. Logistic regression was used to test for genotype associations with cervix cancer status as well as baseline characteristics (age, ethnicity and smoking status) and secondary outcomes such as HIV status and abnormal cytology (Pap smear) in the control group. Statistical analyses were done using Stata 9 software (Texas, USA).

4.3 Results

The observed genotype frequencies for *CASP8* -652 6N ins/del polymorphism did not deviate from HWE for the black controls (P = 0.614) (data not shown here) but deviated slightly in the mixed-ancestry controls (P = 0.045) (data not shown here). Genotyping data for *CASP8* -652 6N ins/del polymorphism was obtained on 445 of the 447 cases and 1221 of the 1407 controls. The frequency of *CASP8* -652 6N del allele was 53% in black cases and 58% in the black controls and 47% in mixed-ancestry cases and 46% in controls of mixed-ancestry. The genotype frequencies for

CASP8 -652 6N ins/del polymorphism did not show statistically significant association (P = 0.948) with cervical cancer (Table 4.1).

Table 4.1 - Association statistics for *CASP8* -652 6N ins/del genotypes for cases and controls.

	Controls (n = 1221)		**Cases** (n = 445)			
	Black	**Mixed-ancestry**	**Black**	**Mixed-ancestry**	**Genotype-cervical cancer association, adjusted for ethnicity and smoking**	
	257 (21)	964 (79)	106 (24)	339 (76)		
Genotypes	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**P-value**	**OR (95% CI)**
***CASP8* -652 6N ins→6N del**						
ins/ins	43 (17)	265 (26)	18 (17)	84 (25)	-	1
ins/del	129 (50)	510 (53)	63 (59)	188 (55)	0.247	0.85 (0.65-1.12)
del/del	85 (33)	189 (21)	25 (24)	67 (20)	0.948	1.01 (0.73-1.41)

P-values and OR (95% confidence intervals) are for test of genotype association with cervix cancer risk, adjusted for ethnicity and smoking. Cases = Women with cancer of the cervix (ICC), Controls = Women without cancer of the cervix, n = counts.

Stratifying into ethnic groups also did not show any significant association of this polymorphism with either black African (P = 0.313, data not shown here) or with women of mixed-ancestry (P = 0.591, data not shown here).

The association of this (*CASP8* -652 6N ins/del) polymorphism was then investigated in the control group with abnormal cytology and high-risk HPV infection. When individuals with abnormal cytology (positive for ASCUS, LSIL or HSIL) were compared to individuals with normal cytology, a statistically significant association (P = 0.048) was found with *CASP8* -652 6N del/del genotype and abnormal cytology (Table 4.2).

Table 4.2 - Association statistics for CASP8 -652 6N ins/del genotypes according to cytology in the control group.

	Normal cytology (n = 969)		**Abnormal cytology** (n = 161)			
						Genotype-cervical cancer association, adjusted for ethnicity and smoking
	Black	**Mixed-ancestry**	**Black**	**Mixed-ancestry**		
	177 (18)	792 (82)	56 (35)	105 (65)		
Genotypes	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**P-value**	**OR (95% CI)**
***CASP8* -652 6N ins→6N del**						
ins/ins	32 (18)	227 (29)	7 (13)	23 (22)	-	1
ins/del	95 (54)	408 (51)	23 (41)	62 (59)	0.165	1.37 (0.88-2.14)
del/del	50 (28)	157 (20)	26 (46)	20 (19)	0.048	1.66 (1.00-2.75)

Abnormal cytology = Positive for pap smear test (i.e. positive for ASCUS + positive for LSIL + positive for HSIL), Normal cytology = Negative for pap smear test.

Comparing the high-risk HPV-infected individuals with non-HPV-infected individuals showed a borderline association (P = 0.058) with *CASP8* -652 6N del/del genotype and high-risk HPV infection (Table 4.3).

Table 4.3 - Association statistics for *CASP8* -652 6N ins/del genotypes for high-risk HPV infection in the controls.

	High-risk HPV negative (n = 933)		**High-risk HPV positive** (n = 197)					
	Black 173 (19)	**Mixed-ancestry** 760 (81)	**Black** 60 (30)	**Mixed-ancestry** 137 (66)	**Genotype-cervical cancer association, adjusted for ethnicity and smoking**		**Genotype-cervical cancer association for Black controls, adjusted for smoking**	
Genotypes	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**P-value**	**OR (95% CI)**	**P-value**	**OR (95% CI)**
***CASP8* -652 6N ins→6N del**								
ins/ins	32 (19)	213 (28)	7 (12)	37 (27)	-	1		
ins/del	94 (54)	400 (53)	24 (40)	70 (51)	0.964	1.01 (0.68-1.49)	0.793	1.13 (0.44-2.90)
del/del	47 (27)	147 (19)	29 (48)	30 (22)	0.058	1.53 (0.99-2.38)	0.030	2.84 (1.11-7.28)

High-risk HPV negative = Negative for Hybrid Capture II HPV Test, High-risk HPV positive = Positive for Hybrid Capture II HPV Test.

When the high-risk HPV infection data was separately analysed in both the black African and women of mixed-ancestry, a statistically significant association (P = 0.030) was found with high-risk HPV infection and *CASP8* -652 6N del/del genotype in black Africans (Table 4.3), but not in women of mixed-ancestry (P = 0.551, data not shown here).

CASP8 -652 6N ins/del polymorphism was not found in LD with FasR-1377G/A (D' = 0.129, LOD = 0.47, r^2 = 0.004), FasR-670A/G (D' = 0.076, LOD = 0.29, r^2 = 0.002) and FasL-844T/C (D' = 0.042, LOD = 0.09, r^2 = 0.001) (Fig. 4.2) while FasR-

1377G/A and FasR-670A/G were in tight LD (D' = 1.0, LOD = 20.87, r^2 = 0.101) (Fig. 4.2) as described in chapter 2.

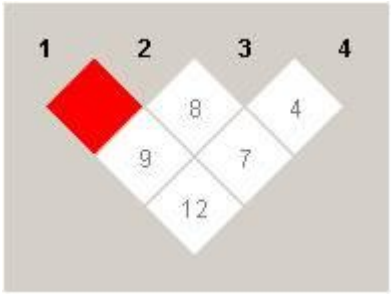

Fig. 4.2: An LD plot showing the r^2 value for the three genes studied in this project.

Marker 1 = FasR-1377G/A, Marker 2 = FasR-670A/G, Marker 3 = FasL-844T/C, Marker 4 = CASP8 -652 6N ins/del. The numbers in the box represent r^2 as a percentage.

The haplotype analysis of *CASP8* -652 6N ins/del with all the three Fas/FasL polymorphisms did not show any association with cervical cancer.

4.4 Discussion

Not much is known about the genetic variations in the *CASP8* gene and their roles in human cancer susceptibility. *CASP8* -652 6N ins/del polymorphism has been associated with several pathological conditions including different types of cancers in different populations with conflicting results (Cybulski et al., 2008;De et al., 2009;Frank et al., 2008;Ji et al., 2009;Li et al., 2008;Ni et al., 2009;Pittman et al., 2008;Sun et al., 2007;Wang et al., 2009;Yang et al., 2008). This polymorphism has also been shown to influence the susceptibility to cervical cancer in one of the studies (Sun et al., 2007). This variant has a direct functional effect on risk of tumour

progression as it decreases *CASP8* transcription and hence reduction in caspase-8 expression (Sun et al., 2007). Biochemical analyses have also shown that T lymphocytes with the deletion variant display reduced caspase-8 activity and reduced AICD when stimulated with cancer cell antigens (Sun et al., 2007).

We are the first to study the frequency of the *CASP8* -652 6N ins/del polymorphism in an indigenous black African as well as in a population of mixed-ancestry. We did not find a significant association of *CASP8* -652 6N del/del genotype and cervical cancer (P = 0.948) when comparing control women of black African and mixed-ancestry origin. The frequency of the *CASP8* -652 6N del/del allele was found to be 26% in Chinese population whereas it was 58% for black African and 46% for mixed-ancestry in our population. It has been previously reported that a mutated form of caspase-8 acts in a dominant-negative manner preventing the recruitment of the wild type forms of caspase-8 to death receptors (Kim et al., 2003;Mandruzzato et al., 1997). This results in a blockage of signal transduction via the death receptor and apoptotic cell death pathway (Kim et al., 2003;Mandruzzato et al., 1997). This is supported by our observation of an increased likelihood of having abnormal cytology (ASCUS + LSIL + HSIL) (P = 0.048) among carriers of the *CASP8* -652 6N del/del genotype compared to individuals with normal cytology. High-risk HPV infection also showed a borderline significant effect (P = 0.058) with *CASP8* -652 6N del/del genotype. However, on separating the two ethnic groups, the black subjects presented with significantly higher risk of HPV infection (P = 0.030) if they were carriers of the *CASP8* -652 6N del/del genotype. As the association of this genotype is not observed with the cervical cancer patients which suggests that majority of the *CASP8* -652 6N del/del genotype carriers clear the HPV infection and the pre-

cancerous lesions regress. This would imply that those with the *CASP8* -652 6N del/del genotype do not progress to cancer of the cervix with a mechanism hitherto unknown. We hypothesize that the shift in roles of *CASP8* -652 6N del/del genotype happens somewhere during the early stages pre- cancer development including HPV infection.

It has been reported that several isoforms of caspase-8 exist, most common of them all is caspase-8 long (caspase-8L) which results from a 136 bp deletion (Mohr et al., 2005). It lacks proteolytic activity and does not facilitate signal transduction from activated death receptors acting in a dominant-negative manner (Fulda, 2009). Thus, caspase-8L distribution in different populations may affect the observed effects of the *CASP8* -652 6N ins/del polymorphism. Its distribution in the current study population is not known. More studies are needed to really tease out what could be the contributory factor in the observed ethnic differences as well as non-association of the *CASP8* -652 6N with cervical cancer, an observation reported by our group. It is important to note that *CASP8* -652 6N deletions, is not the only way caspase-8 expression is lost. A recent report shows that DNA methylation of *CASP8* gene is also used to switch off caspase-8 expression (Wu Y et al., 2010). Thus, we might not have found a difference in the distribution of the *CASP8* -652 6N ins/del between cases and controls but the actual expression levels might be significantly different. Though there is evidence that genetic frequency difference in different races do not generally influence their biological impact on a disease (Ioannidis et al., 2004), the possibility still remains. The presence of another causative mutation in tight LD with this polymorphism can also not be ruled out.

However, it has been shown that the death receptor pathway behaves aberrantly in early stages of precarcinogenic lesions, dysplasia and carcinoma *in situ* (Gratas et al., 1998;Kase et al., 2002). We hypothesize that during early stages of cancer development the caspase-cascade, mediated mainly by the death receptor pathway activates apoptosis in malignant cells. This will eliminate the malignant cells and slow down the process of cancer development. Individuals carrying the *CASP8* -652 6N del/del variant will express less caspase-8 hence less apoptosis of the malignant cells leading to a faster progression of tumour compared to the individuals carrying the *CASP8* -652 6N ins/ins variant of it. Our results showing a significant susceptible effect with abnormal cytology (Table 4.2) and with high-risk HPV infection (Table 4.3) with the deletion variant of *CASP8* (*CASP8* -652 6N del/del) support this hypothesis (Fig. 4.3).

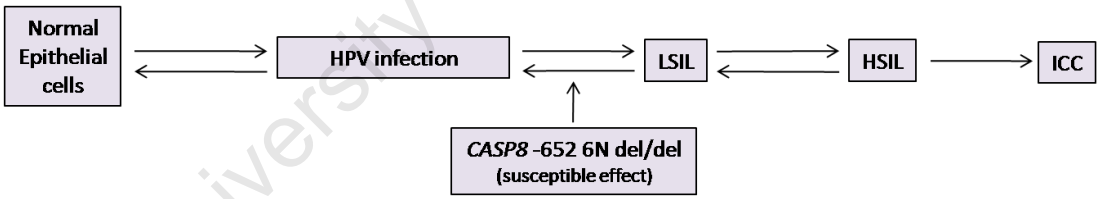

Figure 4.3 - Schematic diagram showing the role of CASP8 -652 6N del/del variant during development of ICC.

4.5 Conclusion

Our study did not show any significant association of *CASP8* -652 6N ins/del polymorphism with cervical cancer, but showed a statistically significant association with abnormal cytology in black African women and women of mixed-ancestry. We also showed a significant effect of this polymorphism with high-risk HPV infection in black African women**.** *CASP8* -652 6N del/del genotype increased the risk of developing abnormal cytology in black African women and women mixed-ancestry and increased the risk of high-risk HPV infection in black African individuals. Further studies are needed with patients at different stages of cervical cancer to confirm our findings.

CHAPTER 5

CCR2-V64I POLYMORPHISM IS ASSOCIATED WITH INCREASED RISK OF CERVICAL CANCER BUT NOT WITH HPV INFECTION IN SOUTH AFRICAN WOMEN

5.1 Introduction

The human immune system releases chemokines that triggers recruitment of local inflammatory and immune cells as one of the earliest responses to HPV infection. CCR2 is one of the major chemokines and a receptor for the MCP-1, produced largely by tumour cells. MCP-1 is responsible for recruiting macrophages to cervical tumours. Macrophages have multifactorial role in tumour progression, with tumour-cytotoxic characteristics in early stages and a supporting role in tumour angiogenesis during late stages. Functional polymorphism at CCR2 gene might influence the recruitment of macrophages to tumours and affect the outcome of the tumour progression. CCR2V64I polymorphism has been well studied in different diseases including cervical cancer as described in the literature review but not within an African population. This chapter focuses on the role of CCR2V64I in cervical cancer in South African women of black and mixed-ancestry origin.

5.2 Materials and Methods

Participants

A total of 1878 subjects comprising 446 women with invasive cervical cancer (106 black African and 340 women of mixed-ancestry) and 1432 controls (322 black African and 1110 women of mixed-ancestry) without cancer of the cervix were recruited. There were 264 (59%) urban cases and 182 (41%) rural cases compared to 718 (53%) urban controls and 632 (47%) rural controls (residency status was not known for 82 controls).

The mean age for black cases was 43.8 yrs (SD 9.2) and for cases of mixed-ancestry it was 46.0 yrs (SD 8.1). The mean age for black controls was 42.3 yrs (SD 9.0) and for controls of mixed-ancestry it was 44.3 yrs (SD 8.4). The HIV infection status was 4.8% for the cases and 4.7% for the controls. No significant differences in age or HIV status were observed between cases and controls (data not shown here). Among 1258 controls for which pap smear results were available, 185 (15%) were abnormal (91 (7%) had SIL (LSIL and HSIL) and of these 45 (4%) had HSIL) and 1070 (85%) were normal. A significant difference in the degree of smoking was found between the cases and the controls ($P = 0.004$) (data not shown here). Subsequently, all the analyses were adjusted for the smoking status along with ethnicity.

Papanicolaou test

Endocervical scrapings were taken from the control women to conduct papanicolaou tests (Pap smears) to check for cervical cytology as previously described (Shapiro et al., 2003). For this study, all ASCUS (atypical squamous cells of undetermined significance), LSIL or HSIL were considered as abnormal cytology.

Extraction of genomic DNA

The genomic DNA was extracted using TotalNucleicAcid Extraction kit for MagNA Pure Compact nucleic acid extractor (Roche Diagnostics, Germany).

Determination of CCR2-V64I polymorphism

The *CCR2-V64I* (rs 3918354) polymorphism was determined by polymerase chain reaction (PCR) using sequence-specific primers (SSP) followed by agarose gel electrophoresis. Two PCR reactions were carried out for each sample using two different forward primers (CCR2-64V and CCR2-64I) and a reverse primer. 130ng of genomic DNA was amplified in a 10-µl reaction mixture containing 10picomoles of each CCR2-V64I primers: F(CCR2-64V), 5'TGGGCAACATGCTGGTCG3' or F(CCR2-64I), 5'TGGGCAACATGCTGGTCA3' and R, 5'TGGAAAATAAGGGCCACAGAC3' (Tang et al., 1999) and 5-µl 2x ImmoMix™ (Bioline). PCR cycle reactions were performed on an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA) beginning with a denaturing step at 95°C for 2.5 min followed by 10 higher-stringency cycles of denaturing at 94°C for 25 s, annealing at 60°C for 45 s and extension at 72°C for 45 s again followed by 21 lower-stringency cycles of denaturing at 94°C for 25 s, annealing at 58°C for 40 s and extension at 72°C for 40 s with a final extension at 72°C for 6 min. The PCR reaction conditions were adapted and modified from Tang J et. al. (Tang et al., 1999).

The amplified PCR products of 413 base pairs (bp) were analysed by running on 1.5% agarose gel stained with ethidium bromide using an O'GeneRuler™ 50bp

DNA Ladder, ready-to-use (Fermentas Inc, Ontario, Canada). The samples with a wild type allele (G) at position 190 of the CCR2 gene amplified the PCR containing CCR2-64V forward primer and showed no products for the PCR containing CCR2-64I forward primer. Likewise, samples with a mutant allele (A) at position 190 of the CCR2 gene did not show any product for the PCR containing CCR2-64V forward primer rather amplified the PCR containing CCR2-64I forward primer. The heterozygous samples showed products for both the PCR containing CCR2-64V as well as CCR2-64I forward primers (Fig. 5.1).

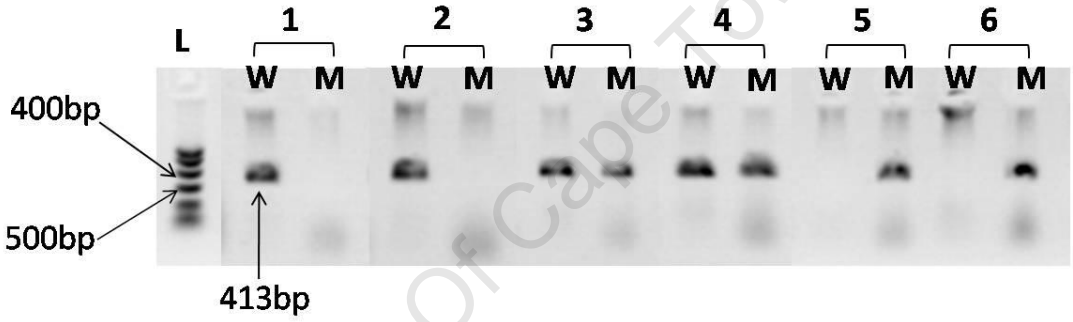

Figure 5.1 - Analysis of the *CCR2-V64I* genotypes on agarose gel.

L = DNA ladder, samples 1 and 2 = GG (CCR2-64V – wild type homozygous), samples 3 and 4 = GA (CCR2-V64I – heterozygous), samples 5 and 6 = AA (CCR2-64I – mutant type homozygous), W = PCR amplifying wild type variant (CCR2-64V), M = PCR amplifying mutant type variant (CCR2-64I).

The genotyping was cross-checked by DNA sequencing of 40 samples, 10 each for wild type, mutant type, heterozygous and 10 randomly selected samples using a forward primer (5'TACGGTGCTCCCTGTCATAAA3') and the same reverse primer previously used for genotyping of CCR2. The DNA sequencing was done using a BigDye Teminator V3.1 Cycle sequencing kit (Applied Biosystems, Foster City, CA) following the manufacturers protocol. Other than the mutant type samples, all the other samples showed 100% confirmation of our genotype results. Due to some ambiguity in the first 10 randomly selected mutant type samples, we decided to

sequence all the mutant samples. Among all the 125 mutant samples (as assigned according to PCR amplification and agarose gel results) only 3 did not show any result in both forward and reverse primer sequencing and were excluded from the final analysis. The rest of the 122 samples showed clear sequencing results of either GG, AG or AA and were scored accordingly.

Statistical Analysis

The genotype distributions were tested for Hardy-Weinberg equilibrium in cases and controls. Logistic regression was used to test for genotype associations with cervix cancer status as well as baseline characteristics (age, ethnicity and smoking status) and secondary outcomes such as HIV status and abnormal cytology (Pap smear) in the control group. Statistical analyses were done using Stata 9 software.

5.3 Results

The observed genotype frequencies for *CCR2-V64I* polymorphism in the controls deviated from Hardy-Weinberg equilibrium (P = 0.001) (data not shown here). This could be due to the fact the our controls were not a randomly chosen population rather handpicked in a 3:1 matched ratio to the cases on decade of age, ethnic group and area of residence (urban/rural). Genotyping data for *CCR2-V64I* polymorphism was obtained on 1378 of the 1432 control specimens. The frequency of *CCR2-64I* variant was 63% in black cases and 24% in the black controls and 63% in cases of mixed-ancestry and 21% in mixed-ancestry controls. A statistically significant, association was found with *CCR2-64I* variant (P = 0.001) and cervical cancer cases and controls after adjusting for ethnicity and smoking (Table 5.1).

Table 5.1 - Counts (n), frequencies (%) and association statistics for *CCR2-V64I* genotypes for cases and controls.

	Controls (n = 1378)		**Cases** (n = 446)			
	Black 305 (n = 22)	**Mixed-ancestry** 1073 (n = 78)	**Black** 106 (n = 24)	**Mixed-ancestry** 340 (n = 76)	**Genotype-cervical cancer association, adjusted for ethnicity and smoking**	
Genotypes	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**P-value**	**OR (95% CI)**
CCR2 G→A						
GG	189 (62)	704 (66)	24 (23)	78 (23)	-	1
AG	112 (37)	356 (33)	81 (76)	255 (75)	0.001	6.22 (4.85-7.97)
AA	4 (1)	13 (1)	1 (1)	7 (2)	0.002	3.99 (1.68-9.50)
AG + AA	116 (38)	369 (34)	82 (77)	262 (77)	0.001	6.14 (4.79-7.86)

P-values and OR (95% confidence intervals) are for test of genotype association with cervix cancer risk, adjusted for ethnicity and smoking. Cases = Women with cancer of the cervix (ICC), Controls = Women without cancer of the cervix.

When the SIL positive (LSIL and HSIL positive) controls (n = 91) were compared with the invasive cervical cancer cases (n = 446) for *CCR2-V64I* polymorphism, a statistically significant association was found with the presence of *CCR2-64I* variant (P = 0.001) adjusted for ethnicity and smoking (Table 5.2).

Table 5.2 - Association statistics for *CCR2-V64I* genotypes for ICC compared with LSIL and HSIL positive controls.

	SIL positive controls (n = 91)		**Cases** (n = 446)			
	Black 31 (34)	**Mixed-ancestry** 60 (66)	**Black** 106 (24)	**Mixed-ancestry** 340 (76)	**Genotype-cervical cancer association, adjusted for ethnicity and smoking**	
Genotypes	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**P-value**	**OR (95% CI)**
CCR2 G→A						
GG	21 (68)	40 (66)	24 (23)	78 (23)	-	1
AG	9 (29)	19 (32)	81 (76)	255 (75)	0.001	7.18 (4.35-11.87)
AA	1 (3)	1 (2)	1 (1)	7 (2)	0.300	2.32 (0.47-11.36)
AG + AA	10 (32)	20 (33)	82 (77)	262 (77)	0.001	6.86 (4.19-11.21)

Cases = Women with cancer of the cervix (ICC), SIL positive controls = Women without cancer of the cervix (ICC) but positive for LSIL and HSIL by pap smear test.

Since the association was found with cancer it was then determined if the same association could be observed in precancers within the control group. The association of *CCR2-V64I* polymorphism with abnormal cytology and HSIL status was investigated only in the control group as the controls represent the larger South-African population. Abnormal cytology was not found to be associated (P = 0.437) (Table 5.3) with *CCR2-64I* variant in the control group adjusted for ethnicity and smoking.

Table 5.3 - Association statistics for CCR2-V64I genotypes according to cytology in the control group.

	Normal cytology (n = 1070)		**Abnormal cytology** (n = 185)			
	Black 210 (20)	**Mixed-ancestry** 860 (80)	**Black** 64 (35)	**Mixed-ancestry** 121 (65)	**Genotype-abnormal cytology association, adjusted for ethnicity and smoking**	
Genotypes	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**P-value**	**OR (95% CI)**
CCR2 G→A						
GG	124 (59)	563 (66)	45 (70)	78 (64)	-	1
AG	84 (40)	287 (33)	17 (27)	40 (33)	0.290	0.83 (0.59-1.17)
AA	2 (1)	10 (1)	2 (3)	3 (3)	0.134	2.28 (0.78-6.69)
AG + AA	86 (41)	297 (35)	19 (30)	43 (36)	0.437	0.88 (0.63-1.22)

Abnormal cytology = Positive for pap smear test (i.e. positive for ASCUS + positive for LSIL + positive for HSIL), Normal cytology = Negative for pap smear test.

Comparing only the HSIL positive samples with all the normal cytology samples also did not show any significant association (P = 0.157) in controls adjusted for ethnicity and smoking (Table 5.4).

Table 5.4 - Association statistics for *CCR2-V64I* genotypes for HSIL in the control group.

	Normal cytology (n = 1070)		**HSIL positive** (n = 45)			
	Black 210 (20)	**Mixed-ancestry** 860 (80)	**Black** 16 (36)	**Mixed-ancestry** 29 (64)	**Genotype-HSIL association, adjusted for ethnicity and smoking**	
Genotypes	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**P-value**	**OR (95% CI)**
CCR2 G→A						
GG	124 (59)	563 (66)	12 (75)	21 (72)	-	1
AG	84 (40)	287 (33)	4 (25)	8 (28)	0.190	0.64 (0.32-1.25)
AA	2 (1)	10 (1)	0 (0)	0 (0)	-	-
AG + AA	86 (41)	297 (35)	4 (30)	8 (28)	0.157	0.61 (0.31-1.21)

HSIL positive = Positive for HSIL by pap smear test, Normal cytology = Negative for pap smear test.

5.4 Discussion

Polymorphisms in the CCR2 gene that alters the macrophage recruitment have been reported to influence a number of diseases including AIDS (Doms and Peiper, 1997;Ioannidis et al., 2001;Mulherin et al., 2003;Smith et al., 1997), multiple sclerosis (Miyagishi et al., 2003), breast cancer (Zafiropoulos et al., 2004), carotid atherosclerosis (Nyquist et al., 2009) and renal transplant rejection (Omrani et al., 2008). This polymorphism has also been associated with cervical cancer in two different populations (Coelho et al., 2005;Coelho et al., 2007;Ivansson et al., 2007a;Zheng et al., 2006).

Persistent HPV infection of epithelial cells is necessary for the carcinogenesis of the uterine cervix, but not all HPV-infected cervical lesions progress to cervical cancer. Chemokines are regarded as an important cofactor in the progression of the cervical lesions to cancer of the cervix (Tindle, 2002).

We are the first to study the frequency of the *CCR2-V64I* polymorphism in an indigenous black African as well as in a mixed-ancestry population. We found a statistically significant association of *CCR2-64I* variant (P = 0.001) with cervical cancer in black African and mixed-ancestry women adjusted for ethnicity and smoking. Our data suggests that women carrying A allele (P = 0.001, OR (95% CI) = 6.14 (4.79-7.86)) and A/A genotype (P = 0.002, OR (95% CI) = 3.99 (1.68-9.50)) at position 190 of the CCR2 gene have an increased risk of cervical cancer compared to women carrying the G variant. When the controls with SIL were compared with cervical cancer cases, it was found that *CCR2-64I* carriers are at greater risk of developing cervical cancer (P = 0.001, OR (95% CI) = 6.86 (4.19-11.21)) from SIL. The analysis of *CCR2-V64I* genotypes in control women with abnormal cytology (P = 0.437) and HSIL (P = 0.157) did not show any statistically significant association. However, the tendencies of the ORs for the above mentioned groups point towards a protective effect of the *CCR2-64I* variant in women with abnormal cytology (OR (95% CI) = 0.88 (0.63-1.22)) and HSIL (OR (95% CI) = 0.61 (0.31-1.21)) compared to women with normal cytology.

Our results showing a susceptible effect of the *CCR2-64I* variant to cervical cancer (comparing controls without cancer of the cervix with controls SIL) are conflicting with reports showing a protective effect of this variant for cervical cancer by two other groups (Coelho et al., 2005;Ivansson et al., 2007a). Also our data showing no association of *CCR2-64I* variant with HSIL when compared to individuals with normal cytology, do not match Coelho et. al. (Coelho et al., 2007) who reported an increased risk of *CCR2-64I* variant with HSIL. The contradictory results might be

due to one or several factors, including the difference in ethnic origin of the population studied the difference in sample size and high percentage of the mutant allele (*CCR2-64I*) in our population. The Portuguese population had a frequency of 12.2% for the CCR264I variant, the Swedish population had a frequency of 13%, whereas in our population the frequency was much higher, (24% in black Africans and 21% in mixed-ancestry). The fact that we did not find any association with abnormal cytology or HSIL in the control group but found a susceptible effect with cervical cancer suggests that *CCR2-64I* variant is not associated with susceptibility to HPV infection or HSIL in our population but increases the risk of ICC at a later stage during the development of cancer of the cervix from HSIL (Fig. 5.2).

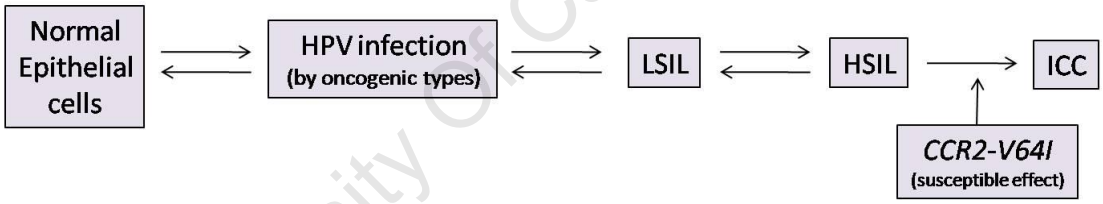

Figure 5.2 - A schematic diagram showing the susceptible effect of CCR2-64I variant during development of ICC.

Our results showing no association of *CCR2-64I* variant with HPV infection with tendencies of the ORs toward a protective effect are in line with Zheng B et. al (Zheng et al., 2006) who did not find any association of *CCR2-64I* but observed similar ORs (OR (95% CI) = 0.006-1.92) as reported by us.

Not much is known about the immune response to HPV-infected epithelial cells as HPV typically does not elicit strong local or systemic immune responses. MCP-1 plays an important role in the development of tumours as it is one of the major

chemokines that induces recruitment of macrophages in tumours including cervical cancer (Vicari and Caux, 2002). Recruitment and activation of macrophages is a vital process for the inflammatory response of the human body. Though macrophages display tumor cytotoxicity, tumor-associated macrophages (TAMs) mainly have protumour functions (Mantovani et al., 1992) and help in tumor angiogenesis. Increased expression of MCP-1 recruits more macrophages which speed up the process of tumour destruction or progression depending upon the type of macrophages recruited. After the infection of epithelial cells by HPV, the MCP-1 expression decreases from LSIL to HSIL and increases again from HSIL to ICC (Riethdorf et al., 1996). Tumor cells have been reported with high levels of MCP-1 expression (Kleine-Lowinski et al., 1999). Macrophages which are recruited by MCP-1 chemokine, express CCR2 on their cell-surface. The *CCR2-64I* variant is associated with increased expression of CCR2A (due to increased stability of CCR2A) on the cell-surface of monocytes. This increases the attraction of monocytes to tumour cells producing MCP-1. In early stages of infection, the increased recruitment of monocytes results in more macrophages and associated cells (DC and NK cells) converging on the developing tumours to destroy the progressing tumours cells. Therefore, increased expression of CCR2 receptors results in increased recruitment of macrophages and possibly faster destruction of a developing tumour. Thus the *CCR2-64I* variant might be associated with reduced risk of developing cancer in the early phase. Our results with HPV-infected individuals do point toward a reduced risk of *CCR2-64I* with abnormal cytology and HSIL positivity when compared to individuals with normal cytology (according to the tendencies of the ORs) though not statistically significant.

However, once the tumour cells evade the immune system, the macrophages that are recruited towards elimination of the tumour switch to TAMs (Lamagna et al., 2006;Lin and Pollard, 2007). The increased stability of CCR2A due to *CCR2-64I* variant sustains the tumours by continuously recruiting TAMs that support tumour angiogenesis. It is not known when this switch from tumour cytotoxic macrophages to TAMs occurs during the development of cervical cancer. We hypothesize that the switch occurs during early phase of HSIL. Thus the *CCR2-64I* variant would be associated with increased risk of cancer in the later stage of tumour development (in this situation after progressing to HSIL) when compared to the *CCR2-64V* variant which is associated with less stable CCR2A stability and expression.

CCR2 gene lies within 10kbp from *CCR5* and are in strong LD with *CCR5*. CCR5 protein is also known to play important roles in inflammatory responses to infection. It has a crucial role in chemotaxis of Th^1 cells and $CD8^+$ effector T cells (Zaitseva et al., 1998). The possibility that the observed genetic association in the *CCR2* gene could be linked to the *CCR5* gene or acting through strong LD by another modifier gene in *CCR5* cannot be ruled out.

5.5 Conclusion

Our study showed a significant association of *CCR2-V64I* polymorphism with cervical cancer, but did not show any association with HPV infection or HSIL. *CCR2-64I* variant showed an increased risk of cervical cancer but not with infection by HPV. This implies that this mutation is associated with a late event in the progression to cervical cancer. Further studies are needed with patients in different stages of cervical cancer to confirm our findings.

CHAPTER 6

CONCLUSION

Genetic diseases are divided into monogenic and complex disorders. Monogenic or Mendelian disorders are caused by a single mutation or more than one mutation in a single gene. Complex diseases, also called non-Mendelian disorders are multifactorial and highly complex in nature. Complex disorders can be caused by both environmental and genetic risk factors interacting in a complicated pattern to produce a particular disorder. Many common diseases are multifactorial such as diabetes, hypertension, allergy, cancer, cardiovascular and psychiatric diseases.

Human-disease gene-identification was mainly limited to monogenic disorders prior to 1980. The invention of polymerase chain reaction (PCR) and mutation screening technologies along with the Human Genome Project (HUGO) to identify human protein coding genes (Collins FS, 2004;Lander et al., 2001) and the International HapMap Project to identify the human genetic variation (Frazer et al., 2007;Gibbs AR, 2005) have moved the field much faster since then. Most of the monogenic diseases are now mapped with their causative gene being identified. On the other hand identifying risk genes in complex diseases are much more difficult as there are many factors involved, including different causative genes influencing with different degrees of severeity. The complex interaction between different genes and between genes and the environmental plays a critical role in the outcome of a complex disorders such as cancer. The present day medical genetics research has moved more towards unravelling the aetiology of complex disorders and to determine different

causative agents and their pattern of interaction with each other to produce a particular disease.

Cervical cancer, a complex and multifactorial disease which is mainly caused by persistent infection with oncogenic types of Human Papillomavirus (HPV) exhibits differential risk in individuals due to the combination of different risk factors mainly genetic and environmental. In order to estimate or measure risk to develop cervical cancer, HPV infection is included as a primary causative agent and outcome of disease is dependent on interactions with other infectious agents such as HSV-2, the variable host genetics involving genes that play major role in immune surveillance, programmed cell death and regulation of cell cycle. It is well established that individual immune response plays a vital role in protection to different viral infections and also to cancer. Host genes play a key role in shaping an individuals immune response that can eliminate a viral infection from the body or stop the growth of the tumourous cells.

The presence of HSV-2 and HPV infections is a significant risk to developing cervical cancer compared to infection with only HPV. The interaction between HSV-2 and high-risk HPV types (mainly HPV-16 and 18) has been suggested to play an important role in the development of cervical cancer (Jones, 1995). Several epidemiological studies have associated HSV-2 infection to an increased incidence of cervical cancer (Jones, 1995).

HSV-2 genome is made up of two distinct and morphologically separate transforming fragments, mtrII and mtrIII that can transform normal human cells to

tumour cells. Studies have demonstrated the ability of mtrIII to co-operate with HPV-16 and HPV-18 to transform human fibroblasts or keratinocytes. PCR analysis of the transformed cells shows consistent presence of HSV-2 DNA sequences (Dhanwada et al., 1992). Human keratinocytes that are immortalized with HPV-16 (FELP) or HPV-18 (FEA) infected with plasmids containing mtrIII results in a higher saturation density cell lines. Only FELP cells (not FEA) developed into benign lesions in *nu/nu* mice (Dhanwada et al., 1993). Genital epithelial cells containing HPV-16 sequences have been shown to convert into tumourigenic squamous cell carcinomas by plasmids containing mtrII (DiPaolo et al., 1990). Follow-up investigation did not find HSV-2 DNA sequences, however, the oncogenic potential of HSV-2 was not lost. These studies support the fact that infection with high-risk HPV types is necessary but not sufficient for cervical cancer and HSV-2 infection may play a role in the outcome of the disease in association with HPV.

Apoptosis is one of the most important mechanisms of the human immune system to control viral infections and malignant cells. It is a highly programmed process of cell suicide. Apoptosis induced in viral infected cells helps to eliminate a viral infection. On the other hand, apoptosis induced in malignant cells helps to restrict uncontrolled growth of the cancerous cells. Therefore, appropriate regulation of apoptotic process is extremely crucial in controlling a viral infection as well as spread of a cancer. The signals produced by the cell-surface death receptor Fas together with FasL activate caspase-8 which triggers apoptosis. Alterations in the genes involved in this cell death pathway are bound to manipulate the regulations of the process of apoptosis thereby influencing the outcome of viral infections or spread of cancerous cells.

Hence, functional polymorphisms at genes encoding Fas, FasL and caspase-8 fall under scrutiny in understanding the risk factors contributing to persistent HPV infection and different stages of pre-cancerous lesions leading to cervical cancer. *CASP8* -652 6N del/del genotype significantly increased the risk of abnormal cytology in South African women of black and mixed-ancestry origin. The same CASP8 genotype also increased the risk of high-risk HPV infection in black African individuals.

FasR-1377A allele and FasR-1377/FasR-670AG haplotype significantly reduced the risk of HSV-2 infection with a mechanism hitherto unknown. To our knowledge, this is the first non-HLA host genetic association of HSV-2 infection reported till now.

Recruitment and activation of macrophages is an important process of host immune response against tumour development. The macrophages play a dual role in development of cancer from the early stages of very few pre-cancerous cells. At the beginning of pre-cancerous lesions macrophages have tumour-cytotoxic charastiristics which helps to eliminate the cancerous cells. At later stages when tumour cells have evaded the immune system the macrophages switch to tumour-angiogenesis and aid the tumour to grow faster. CCR2 is a major receptor for MCP-1 that recruits macrophages to tumour cells. Altered CCR2 expression due to functional polymorphism on the CCR2 gene would influence the recruitment of macrophages thus affecting the growth of the tumour cells. *CCR264I* variant significantly increased the risk of cervical cancer but did no affect the risk of HPV infection or HSIL pointing to a late effect of the polymorphism in development of

cervical cancer. This polymorphism may be used as a target to manage cervical cancer.

To summarise our findings on host genetic determinants of HPV infection, and cervical cancer South African women; different genes influence HPV infection and pre-cancerous lesions leading to cervical cancer differently. Figure 6.1 captures the essence of our findings in HPV infection and cervical cancer in a simplified and summarised form.

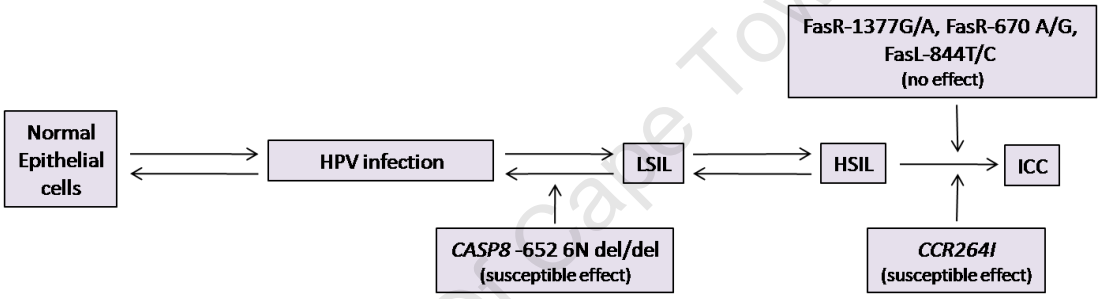

Fig. 6.1: A schematic diagram showing the role of Fas, FasL, CASP8 and CCR2 gene polymorphisms during development of ICC

This is the first time the roles of Fas, FasL, CASP8 and CCR2 gene polymorphisms have been studied in HPV infection and cervical cancer in indigenous black and mixed-ancestry African population. Our finding of an association of a Fas polymorphism with HSV-2 infection is a first time non-HLA host genetic determinants for HSV-2 infection have been reported.

The limitations of these studies can also not be ignored. Although the strength of the studies lie in selection of a well matched cohort for cervical cancer, it should be acknowledged that the study was not initially designed to investigate host genetics. Thus the lack of homogeneity comes for the population group selected for the work.

It is well known that a genetically mixed group is never an ideal population to do genetic studies. Though the black African group is a homogenous group, the mixed-ancestry population is mixed with people from diverse geographical locations varying from Asia to Africa and Europe. Hence the chance of finding a not so strong genetic modifier becomes less probable for a mixed group. The study finding association between genotypes and HSV-2 infection deals with the control subjects for the cervical cancer case-control cohort. This is again a limitation, as the HSV-2 study lacked a well designed cohort with perfectly matched cases and controls as in all the other cervical cancer studies. The possibility of false positive values from genotyping results is always a concern. However, the large sample size used in this study might have reduced the effect of false positive values.

Keeping these limitations in mind, it is important to further investigate the polymorphisms investigated in this thesis in a homogenous and well matched case-control cohort. Probably an SNP tagging approach to a wide range of candidate genes or a whole genome scan would drive the search for the causative genetic effect to cervical cancer faster. The host genetics research of complex and infectious diseases have created a lot of interest in the recent past. This is important in better understanding of the infectious disease biology, the host-virus interaction and the way the host responds to that. Further research in this field will also enable the researchers to design genetic markers for diagnostic purposes for infectious diseases. That will help for better prognosis and management of these diseases.

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