Biomolecular and epidemiological aspects of human papillomavirus induced cervical carcinogenesis

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Biomolecular and Epidemiological Aspects of Human Papillomavirus Induced Cervical Carcinogenesis

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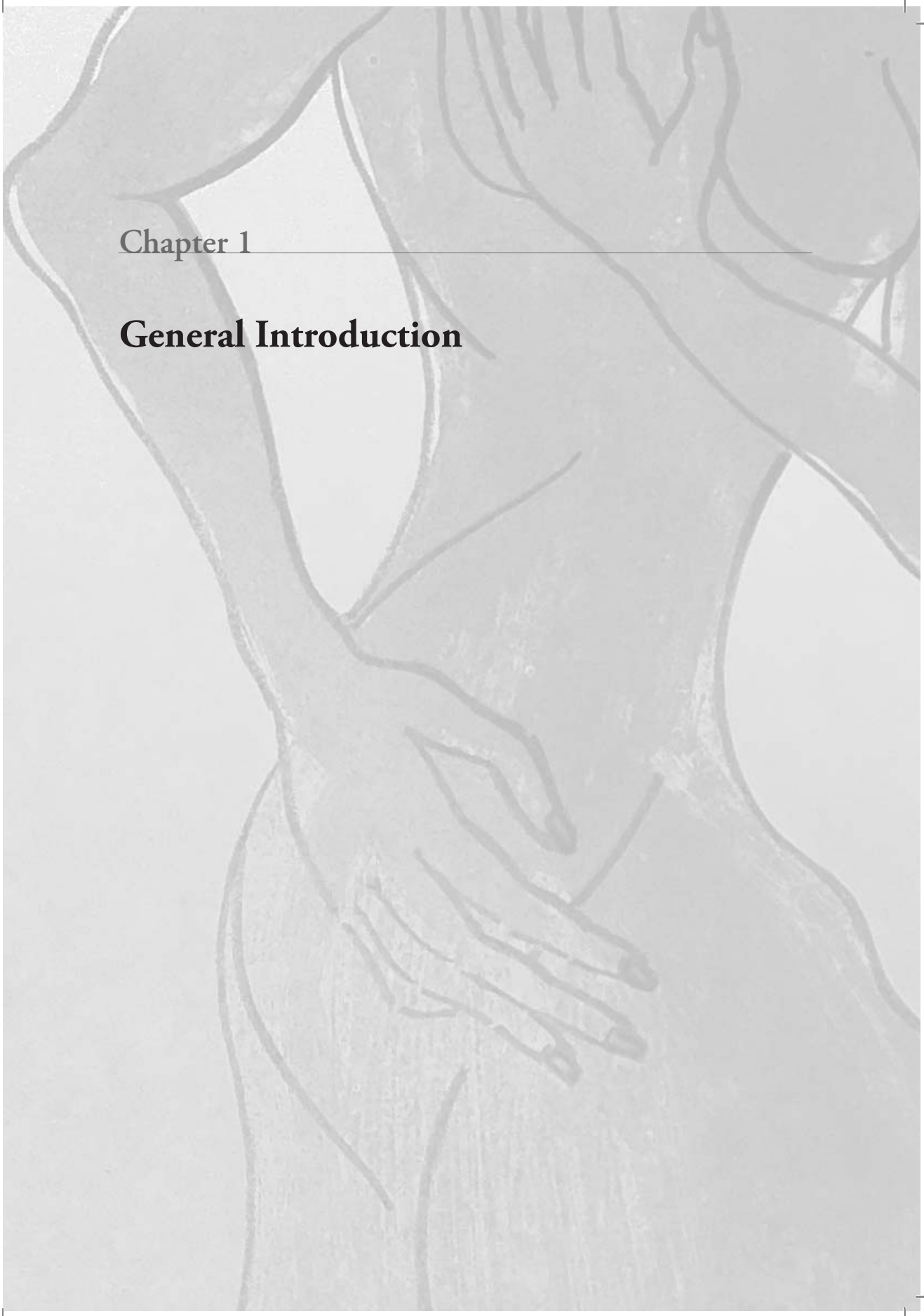

Chapter 1

General Introduction

Contents

1 Cervical Cancer

Aetiology

In 1842 Rigoni-Stern first mentioned that sexual intercourse and cervical cancer appeared to be related, because the disease was rare in nuns and common in prostitutes[1,2]. The idea that nuns, virgins and spinsters will not develop cervical cancer, despite being actively passed down through decades, was never scientifically well underpinned[3]. Nevertheless, epidemiological studies did show that cervical carcinoma was related to promiscuity and a young age of first sexual contact[4-6]. In 1976, Harald zur Hausen suggested that the development of cervical carcinoma was influenced by the sexually transmittable human papillomavirus (HPV), a virus until then only known to cause genital warts[7]. Several years later he first isolated, characterised and cloned HPV DNA from genital warts together with De Villiers and Gissman[8,9]. Since then, infection with human papillomavirus has been found to be the aetiological agent of cervical cancer[10-13]. The extensive HPV-mediated (cervical) carcinogenesis is elegantly investigated and summarised by Steenbergen[14].

Cervical carcinogenesis is a multistep process in which HPV infection is a necessary and early event. Other important steps are genetic changes and a failing immune system, which will be discussed in more detail in the following paragraphs.

Clinicopathology

The cervix uteri consists of the ectocervix and the endocervix, anatomically divided in the visible part (ectocervix) and the non-visible part (endocervix) of the cervix. The ectocervix is mainly lined with non-keratinizing stratified squamous epithelium and the endocervix with mucus producing columnar epithelium. The squamocolumnar junction (SCJ) is defined as the border between the two epithelia. In premenstrual women the SCJ is often located in the cervical canal, in the fertile years the SCJ is mostly located on the ectocervix. A physiological process called squamous metaplasia occurs in the cervix and arises from the subcolumnar "reserve cells". During this process columnar epithelium is gradually replaced by squamous cell epithelium. The SCJ shifts cephalad and in post-menopausal women it is located in the endocervix again. The area where the squamous metaplasia has taken place, which is the area between the original and the new SCJ, is called the transformation zone (**FIGURE 1***)*. The cells in this transformation zone are less stable and therefore particularly susceptible to viral infections. It is in this area where cervical carcinogenesis usually occurs[15-17].

A disturbed proliferation of squamous cells is called dysplasia or cervical intraepithelial neoplasia (CIN) and is the precursor of invasive carcinoma. The grading of CIN is based on the severity of the changes and especially on the proportion of the epithelial layer with neoplastic changes. In CIN I a third, in CIN II two third and in CIN III (almost) the total layer of epithelium contains atypical cells. Although CIN is a precursor lesion, the majority

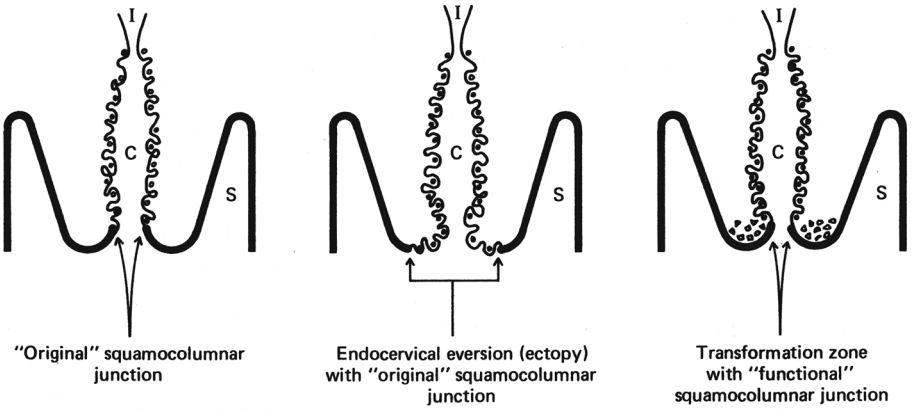

FIGURE 1
Squamocolumnar Junction and Transformation Zone
Adapted from[155]

of the untreated mild dysplasias persist or regress to normal cytology. The likelihood of progression of CIN I, CIN II and CIN III to invasive carcinoma ranges from 0.4 to 1%, 1.2 to 5%, and 3.9 to greater than 12%, respectively[18-20].

Different clinical (sub)stages of invasive cervical cancer are defined by the Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) as summarised in **TABLE 1**[21,22].

Several biological types of primary cervical neoplasms exist. Squamous cell carcinoma accounts for almost 80%, adenocarcinomas and adenosquamous carcinomas for most of

TABLE 1
FIGO stages, the different clinical (sub)stages of invasive cervical cancer as defined by the Fédération Internationale de Gynécologie et d'Obstétrique[21,22]

STAGE	SUBSTAGE
FIGO I – Limited to the uterus	**IA** - diagnosed only by microscopy • IA1 – stromal invasion < 3mm + ≤ 7mm spread • IA2 – stromal invasion 3-5mm + ≤ 7 mm spread **IB** - lesion with invasion > 5 mm or > 7mm spread • IB1 – lesion ≤ 4cm in greatest dimension • IB2 – lesion > 4 cm in greatest dimension
FIGO II – Invades beyond uterus	**IIA** – without parametrial invasion **IIB** – with parametrial invasion
FIGO III – extends to pelvic wall/ lower 1/3 vagina	**IIIA** – involves lower 1/3 of vagina **IIIB** – extends to pelvic wall and/or causes hydronephrosis or non-functioning kidney
FIGO IV	**IVA** – invades mucosa of bladder or rectum and/or extends beyond true pelvis **IVB** – distant metastases

the remaining 20%. Very rare types of epithelial tumours of the cervix are, for instance, glassy cell carcinoma and small cell carcinoma[23].

Treatment and Prognosis

The diagnoses CIN III or less depend on pathological findings. CIN III is treated by destruction or removal of the whole transformation zone. When the tumour is invasive the treatment of cervical carcinoma depends on its clinical (FIGO) stage. A uterus extirpation is usually the therapy of choice in case of micro invasive carcinoma (stage IA). When there is a wish for fertility in a woman with cervical cancer stage IA1 conisation is an option. In FIGO stage IB and IIA a radical uterus extirpation with (pelvic) lymphadenectomy or radiotherapy is performed. A more accurate staging of the tumour and estimation on prognosis is possible with surgical treatment. In addition, surgery permits the ovaries to be spared, which prevents fertile women from entering the menopause prematurely. A third advantage is the decrease in problems with sexual intercourse, possibly even less frequently arising if the radical surgery is nerve-sparing[24,25]. Postoperative radiotherapy is indicated with positive lymph nodes or positive surgical margins and parametrial involvement. In most clinics postoperative radiotherapy is also performed when other unfavourable prognostic factors are present, consisting of depth of tumour infiltration, lymphovascular space involvement or tumour volume. After randomised clinical trials the NCI now advises to treat the advanced stages (IIB-IV) and high-risk early stages with concomitant chemotherapy and radiotherapy[26].

Early stage cervical carcinoma can be treated successfully in the majority of the cases, with a 5-year recurrence-free survival (RFS) rate of 70-100%[26-28]. Survival for the more advanced stages varies and is influenced by lymph node involvement. The 5-year RFS is 50-70% for stages IB2, IIA and IIB, 30-50% for stage III and falls rapidly to 5-15% for stage IV[26]. Therapy for recurrent cervical cancer is generally disappointing and depends on previously performed radiotherapy. Less than 5% of these patients survive 5 years[26].

The most significant prognostic factor on survival is the FIGO stage, but other significant prognostic indicators exist as mentioned above[27-31]. In addition, a major prognostic factor is the level of development and poverty of the area in which the patient resides. The vast majority of the patients with cervical cancer cannot benefit from the advances of the last decades in treatment of this disease, because they live in impoverished countries with limited resources and no or inadequate screening programmes[26].

Epidemiology

Cervical cancer is the second most common cancer among females worldwide. Over 493,000 new cases are diagnosed yearly and it remains one of the leading causes of death from cancer among women[32,33]. The highest incidence rates are found in developing countries with age adjusted incidence rates up to 68.6 per 100,000 women[32]. In developed

countries the incidence rates have dropped to age standardised rates between 4.3 and 13.5[32,34-36]. In the Netherlands, an example of a low-risk country for cervical cancer, the age adjusted incidence and mortality rates per 100,000 women are 7.3 and 2.3, respectively[32].

The past decades both the cervical carcinoma incidence as well as the occurrence of the advanced FIGO stages have decreased 30-60% in developed countries. Screening programmes in developed countries might account for the majority of this decline in cervical carcinoma incidence and mortality rates although the impact has never been studied in randomised trials[35-37].

Prevention

Prevention of cervical cancer can be accomplished by implementing well organised, population-based screening programmes. The present screening programmes aim to trace cervical precursor lesions by cytologically analysing cervical smears. Several classification systems exist for recording cytological abnormalities, including the Bethesda System[38] and the Papanicolaou Classification[39] (**TABLE 2**). In the Netherlands cervical cytological abnormalities are graded using the KOPAC system, the official Dutch microscopical coding system[40,41]. This system allows for simultaneously scoring of inflammatory and (pre) neoplastic changes. A Pap score is given for communication with clinician and patient.

Nowadays, the developed countries all have effective screening programmes with a coverage and attendance of 50-80%[36]. In most of these countries a cervical smear is taken every three or five years and targets women aged between 30 and 55. Developing countries remain high-risk areas for cervical cancer. They account for 79% of the cervical cancer incidence worldwide and advanced FIGO stages are still of frequent occurrence in these countries[35,36]. Therefore, implementation of screening programmes in developing countries seems an appropriate measure to decrease the high incidence.

TABLE 2

Description, various classification systems and translation of codes for normal squamous epithelial cells and (pre)neoplastic changes

DESCRIPTION	PAPANICOLAOU	BETHESDA	KOPAC P-Code
Normal	Pap I	Normal	P1
Borderline Changes	Pap II	ASCUS	P2-3
Mild Dysplasia	Pap IIIA	(L)SIL	P4
Moderate Dysplasia	Pap IIIA	(H)SIL	P5
Severe Dysplasia	Pap IIIB	(H)SIL	P6
Carcinoma in Situ	Pap IV	(H)SIL	P7
Micro invasive Carcinoma	Pap V	Carcinoma	P8
Squamous Cell Carcinoma	Pap V	Carcinoma	P9

Cervical Cancer in Suriname

Suriname is a high-risk area for cervical carcinoma with an incidence of 27 per 100,000 women[32]. A three- to sixfold higher percentage of the advanced FIGO stages (IIB-IV) is established compared to the Netherlands, a low-risk country for cervical cancer. There are various ethnicities living in Suriname, which have different cervical carcinoma incidence rates[42]. These ethnicities are the Creoles, the Hindustani, the Javanese, the Maroons, the Amerindians, the Chinese and all possible mixtures of these ethnicities. Hitherto, the high cervical cancer incidence in Suriname and other high-risk countries is attributed to absence of an organised screening programme, a presumed high(er) prevalence of the human papillomavirus (HPV), immunological factors and environmental or cultural based factors, but more research is still needed.

2 Human Papillomavirus (HPV)

Biological Aspects

(Human) Papillomavirus is a genus of the family Papovaviridae. The HPV virions are non-enveloped and icosahedral with a circular double stranded DNA (dsDNA) genome of almost eight kilo bases in length. The dsDNA consists of six open reading frames (ORF) encoding early (E) proteins, two ORFs encoding late (L) proteins, and a non-coding long

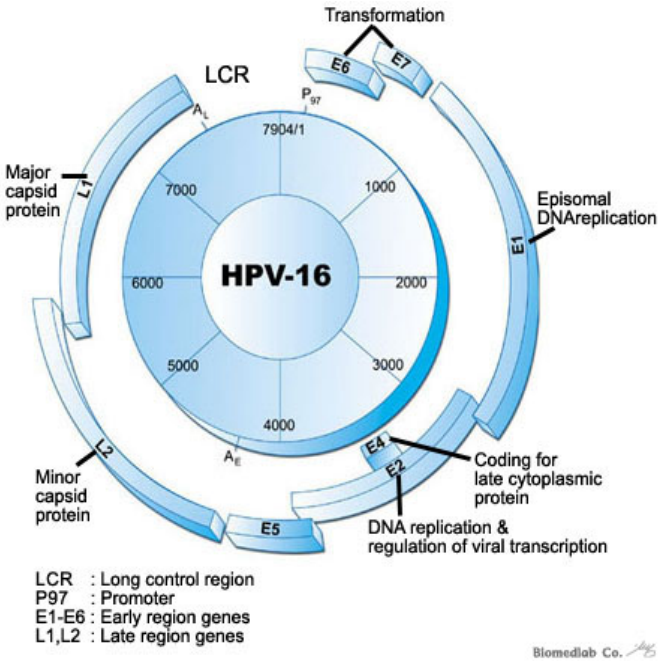

FIGURE 2
The HPV Virion

control region (LCR) (**FIGURE 2**). Papillomaviruses are classified based on their degree of DNA homology in the nucleotide sequences of E6, E7 and L1 ORFs.

Cervical HPV infection occurs through microabrasion of the genital epithelium allowing access of the viral particles to target cells. For a lesion to persist, it is suggested that the virus has to infect an epithelial stem cell[43-45]. It is generally thought that expression of the viral E1 and E2 proteins maintains the HPV DNA as an episome and facilitates the correct segregation of genomes during cell division[45-47]. The major viral oncoproteins E6 and E7 have been shown to play a vital role in viral episome persistence by interfering with the cell cycle[48,49]. They can stimulate cell cycle progression and associate with cell cycle regulators[50-52]. E6 binds to p53 and herewith inactivates p53-mediated growth suppression and apoptosis[53], whereas E7 binds to pRb which inactivates this negative regulator of the cell cycle[54].

To date, 118 papillomaviruses (PVs) comprising of 96 human and 22 animal papillomavirus types have been completely described and several hundred putative new PVs types are partially characterised[55-58]. The HPV genotypes can be divided into a subgroup

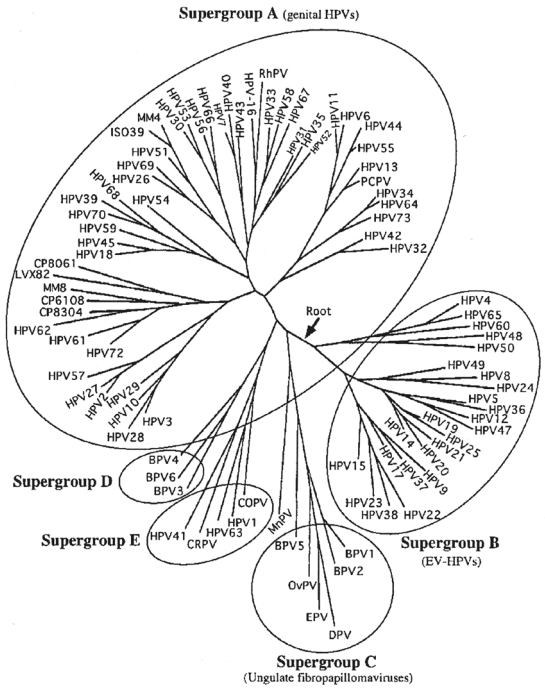

FIGURE 3
Phylogenetic Tree of Papillomaviruses
Adapted from[156]

of mucosal HPV types, which is associated with anogenital lesions and a subgroup of cutaneous HPV types, which induce mostly benign skin lesions. The mucosal HPV types are further classified as (probable) oncogenic or high-risk types which are predominantly found in CIN III lesions and anogenital cancers, and low-risk HPV types which are mainly found in benign and CIN I-II lesions[57-59](**FIGURE 3**). Forty types infecting the anogenital tract are found in anogenital cancer specimens[56,60,61].

Occurrence

Most women undergo an HPV infection during life, but are able to clear it without ever having any clinical symptoms. HPV DNA is detectable in 2% to more than 20% of the global female population at any time[33]. In women with normal cytology or mild dysplasia the predominant HPV types are low-risk. With increasing severity of dysplasia the overall HPV prevalence also increases and the oncogenic HPV types become more prevalent. Finally, in invasive cervical carcinoma the oncogenic HPV prevalence is established to be almost 100% and thus HPV is accepted to be a necessary cause[10-13,33,62-64].

The prevalence and distribution of HPV genotypes show considerable geographic and ethnical variation, especially for the less common types. In most areas the predominant HPV genotypes in cervical cancer are HPV 16 (30-50%) and HPV 18 (10-15%). In non-western countries other types, like 45, 52 and 58, are also detected in a considerable proportion of the cervical cancers[65,66].

It is possible to have an HPV infection with multiple HPV genotypes simultaneously. Different studies report about multiple HPV infections in cervical samples with normal cytology or atypical squamous cells of undetermined significance (ASCUS) and mild to severe dysplasia[67-72]. It is generally thought that the cells infected with the most oncogenic type will eventually transform into the invasive tumour clone. In the majority of invasive carcinomas mainly single HPV infections were detected and until recently only occasionally a multiple HPV infection was found. Because of newly developed techniques better suitable for detection of multiple HPV types, it is now possible to get an accurate indication of their prevalence in cervical carcinoma and its precursors.

Detection Techniques

The (human) papillomaviruses can only replicate in differentiating stratified squamous epithelium, which cannot be grown as a conventional cell culture. Serological tests for HPV have an estimated sensitivity of only 50% using detection of HPV DNA as a standard[73]. Therefore HPV infection and typing can only be accurately diagnosed by molecular methods[73,74]. Several HPV assays are described, but nowadays the polymerase chain reaction (PCR) based techniques are the method of choice due to the greater sensitivity and technical facilities[75]. Since there is significant sequence variation between the genotypes, either a large number of type-specific PCRs or a single broad-spectrum PCR primer set,

can be used. Several general PCR primer sets have been developed, which aim at the most conserved sequences of the viral genome, permitting amplification of a broad spectrum of HPV genotypes[70,73,76-78]. After the HPV detection, the HPV genotyping is performed by sequence analysis, a reverse hybridisation assay[71,79-81] or, more recently, micro arrays[82,83].

3 Immunology and Cervical Carcinoma

Human Leukocyte Antigen (HLA)

Background

The immune system is the specific defence mechanism against the external world. It comprises the antibody mediated (humoral) system, for which B-cells are responsible, and the cellular system, predominantly mediated by cytotoxic T-lymphocytes (CTLs). Both systems are involved in the immunological management of a viral infection. The humoral immune system probably is important for prevention of viral infections, the cellular immune system for the elimination of a virus and virus induced lesions. Immunological surveillance in HPV associated lesions is thus performed by CTLs, which are activated when foreign (antigenic) proteins are presented to the CTL receptor by human leukocyte antigen (HLA) class I. HLA class I molecules are expressed on virtually all cells[84].

HLA

The major histocompatibility complex (MHC) is located on the short arm of chromosome 6 at 6p21.3 and comprises 240 different gene loci[85], of which many encode for HLA molecules. The MHC can be subdivided into three closely linked multigene families, class II (HLA-DR, -DP and -DQ genes), class III (includes genes encoding complement and tumour necrosis factor (TNF)) and class I genes (the classical class IA genes, HLA-A, -B and -C, and the non-classical class IB genes, HLA-E, -H, -G and -F) (**FIGURE 4**). The MHC genes all encode for proteins that control the immune responses to pathogens, graft acceptance or rejection and tumour surveillance. The HLA class I and class II molecules are encoded by, respectively, class I and class II genes. On each chromosome 6 the genes in the class I-III regions compose a combination, called a haplotype. The two haplotypes on the chromosome 6 pair combined are called the HLA genotype. The HLA genotype is expressed as HLA class I and class II molecules on the cell surface and this is called the HLA phenotype.

HLA Class I Antigen Processing and Presentation

HLA class I molecules are expressed on nearly every somatic cell[84] and on virally infected tumour cells. They consist of a polymorphic heavy α chain (HC), encoded by the HLA class I genes HLA-A, -B and -C on chromosome 6p21.3, in non-covalent association with

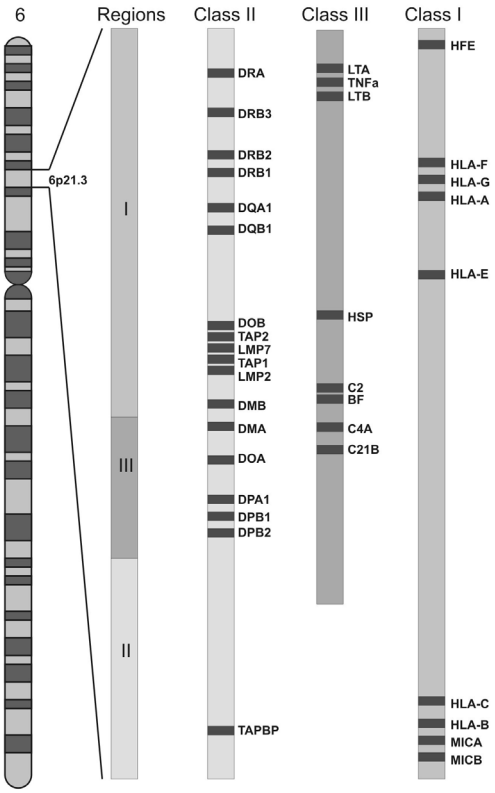

FIGURE 4
The Major Histocompatibility Complex. Provided by E.S. Jordanova

the light β chain, encoded by the β_2-microglobulin (β_2m) gene on chromosome 15q21. The association with β_2m is important for the stability of the HLA class I molecule[86].

The antigen processing and presenting by the HLA class I molecule or HC-β_2m complex concerns mainly endogenous processed antigens (viral or tumour associated products, or waste products from the cell itself). Endogenous proteins are degraded in the cytosol into smaller fragments, called peptides. These peptides are subsequently transported by the transporter associated with antigen processing (TAP), which consists of two subunits TAP1 and TAP2 that form a channel in the endoplasmatic reticulum (ER)-membrane[87]. In the ER, the assembly of the HLA class I heavy chain, the β_2m light chain[88] and the peptides[89,90] is chaperoned by several proteins[91-95]. The newly formed complex is then transported via the Golgi network to the cell surface and is subsequently presented to circulating CTLs (**FIGURE 5**). In addition, TAP independent mechanisms have been described[96-101].

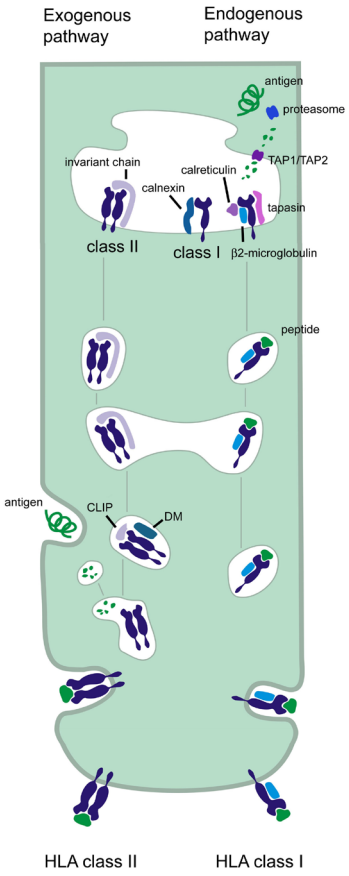

FIGURE 5
HLA Class I Antigen Processing and Presentation. Provided by E.S. Jordanova

Immune Evasion in Cervical Cancer

Loss of HLA class I cell surface expression, HLA class I downregulation, occurs in various solid tumours and tumour cell lines[102,103] and is thought to result in escape from the cytotoxic CTL attack. It occurs frequently in cervical carcinoma and is predominantly caused by losses at chromosome 6p21.3, the region where the HLA genes are localised[104-107]. HLA class I expression was also tested in CIN-lesions with varying outcomes[108-110], but in these lesions knowledge remains limited about altered HLA class I expression in association with the underlying mechanisms. HLA class I downregulation is frequently associated with impaired TAP expression[111-114] and it has been correlated with TAP gene regulatory abnormalities and mutations in various tumour types[115-120].

Currently, research concerning prevention and treatment in cervical cancer focuses on HPV vaccines[121-124]. Therapeutic vaccines are based on the viral oncogenic proteins E6 and E7 and aim to induce cell-mediated responses to eliminate the transformed tumour cells[124]. CTLs will only recognise viral peptides if HLA class I molecules present them on the surface of the infected cell. Therefore, HLA class I downregulation would compromise the effectiveness of an HPV vaccine.

Genetic Basis of Immune Evasion

During carcinogenesis multiple genetic events take place involving proto-oncogenes and tumour suppressor genes (TSGs), two classes of genes that are both involved in tumour progression and metastasis[125-128]. Vogelstein and Kinzler described the multistep nature of cancer[125], which is distinctly illustrated by the multigenic model for colorectal tumorigenesis[129]. It was suggested that only a subset of genetic pathways can initiate the tumorigenic process in particular cell types and that mutation at some genes confers a selective growth advantage[125]. In cervical cancer the two HPV-encoded oncoproteins E6 and E7 can independently induce chromosomal abnormalities, which causes genomic instability and ultimately facilitates carcinogenic progression[130,131].

Alfred Knudson advanced his "two hit" model in 1971 as a necessary condition for certain cancers to develop[132]. All chromosomes exist in pairs and carry the genes, of which most have two similar copies. An alteration in each of two gene alleles inactivates a tumour suppressor gene, leading to tumour development and growth. One hit is an innate (germ line) mutation (occurs in hereditary cancer) or a somatic mutation (in sporadic cancer), the other hit an event that often leads to loss of heterozygosity (LOH)[133-137]. Such an event can be deletion, gene conversion, (mitotic) recombination, translocation, nondisjunction or chromosome loss, chromosome duplication and promoter methylation and could lead to haploinsufficiency[128,137-139]. LOH can be detected by polymorphic repeat markers flanking the locus of interest, or situated in the target gene. Those polymorphic markers are formed based on repeat sequences in the DNA, which are heterozygous for the two gene alleles in a large percentage of the population. The LOH analysis is used to indicate loci that may contain a TSG. However, accurately defining a common LOH region with a possible TSG can be confounded by deficient LOH detection, genetic instability and inter-/intratumour heterogeneity[139].

LOH at chromosome 6p21.3, the region where the HLA genes are located, occurs at high frequencies in cervical cancer[140-146]. With most genes both alleles need to be switched off to inactivate the gene. HLA genes are co-dominant therefore switching off one gene allele could induce inactivation. Koopman *et al.* proved in a study on fresh tumour tissue that LOH at 6p21.3 represents an important and common mechanism by which HLA genes and their products are abolished[107]. LOH on 6p21.3 is also frequently detected in high grade CIN-lesions, indicating that it is an early event in the cervical carcinogenesis[147,148]. A

genetic basis was shown for most of the cervical tumours with an altered HLA phenotype. This involved, besides LOH, class I gene mutation (on chromosome 6p21.3) and β_2m mutation (on chromosome 15q21) or a combination of these events[107,149]. Further investigations are yet needed of the unexplained HLA class I phenotype alterations to clarify the underlying mechanisms.

Other mechanisms causing HLA class I downregulation could be mutation(s) and LOH in the genes encoding for TAP I or II. A recent cervical carcinoma study reported possible mutations in these TAP encoding genes, but the method of detection was not conclusive. In fact, LOH and polymorphisms in TAP genes were studied and loss of TAP expression was not investigated[150].

Vaccination

The close relationship between viral infection and cancer makes HPV an attractive target for prophylactic and therapeutic vaccine development. Prophylactic vaccines are developed to prevent infection by generation of antibodies to recombinant capsid proteins L1 (and L2) that neutralise viral infection[121,122,124,151]. Therapeutic vaccines generally target E6 and E7 which are critical for the immortalisation in (pre)malignant cells in order to induce regression of established infection and possibly control the HPV-associated lesion[121,123,124,152]. The vaccines can be delivered directly as protein, as DNA that encodes and expresses the requisite viral protein(s), or by heterologous viral vectors[153]. Various approaches are being taken in the development of prophylactic HPV vaccines, the most advanced and promising being the use of non-infectious recombinant virus-like particles assembling from pentamers of the L1 capsid protein and inducing high titres of virus-neutralising antibodies[124]. Encouraging results from animal and human vaccine trials have led to large scale efficacy trials concerning prophylactic and therapeutic vaccination[121-124,151]. Recent research on safety and efficacy of candidate prophylactic vaccines have shown a nearly 100% protection against the development of (high-grade) HPV 16 and 18 induced cervical lesions[124,151,154]. Several therapeutic vaccines have been developed and are currently under clinical evaluation[124].

4 Scope of this Thesis

As discussed previously, cervical cancer is preceded by several stages of precursor lesions. Population-based screening programmes aim to trace these precursor lesions by cytologically analysing cervical smears. The premalignancies are mainly induced by HPV infection, which is very common in young women worldwide and influenced by endogenous and environmental factors. Behavioural factors like lifestyle and viral characteristics are important environmental factors. Most HPV infections are transient and are cleared within months as a result of an effective host immune response. Clearance of oncogenic

HPV infection is accompanied by cytological regression, which occurs in the majority of mild cervical abnormalities. The cellular immunity is an important effector mechanism for the clearance of established HPV infection and thus it is likely that the immunological surveillance by CTL responses plays a role in the protection against the development and progression of cervical lesions. CTL responses are generated when foreign (antigenic) proteins are presented to the CTL receptor by HLA class I molecules. TAP is physically associated with HLA class I molecules and is required for the transport and processing of the viral or tumour antigens degraded to peptides.

HLA or TAP aberrations might lead to a failing immunological surveillance, which allows for the oncogenic HPV infection to become persistent. Persistent infection with oncogenic HPV types is essential for the development and progression of cervical dysplasia and, finally, for the development to cervical cancer. It is accepted that HPV is present in all cervical carcinomas, which could be in episomal and integrated form. Viral integration of the HPV in the human genome appears to increase with progression to cervical cancer, but the biological significance is still debated. Occasionally cervical carcinoma is infected with multiple HPV types. Limited knowledge exists of multiple HPV infections in cervical cancer and it is complicated to investigate due to technical difficulties.

The past decades both the cervical carcinoma incidence as well as the occurrence of the advanced FIGO stages have decreased in developed countries. This is predominantly due to the implementation of well-organised screening programmes. The population based screening selects women at risk of developing cervical cancer and prevents it by treating women with moderate and severe dysplasia. In addition, it allows for downstaging of the disease by capturing cervical carcinoma patients in the presymptomatic stages. Unfortunately, cervical carcinoma remains the major cause of cancer related mortality among women in developing countries. Implementation of screening programmes in developing countries therefore seems an appropriate measure to decrease the high incidence.

In **CHAPTER 2** we analysed cervical smears of four different Surinamese ethnicities to determine the prevalence of cytological abnormalities of women attending the first organised screening programme in a high-risk area for cervical cancer. In addition, we investigated whether the differences in cervical cancer incidence existing between the studied ethnicities was reflected in the proportions of cytological abnormalities.

It is valuable to obtain insight in the relative influence of endogenous and environmental factors on differences in cervical carcinoma incidence rates between high- and low-risk areas. This could be achieved by comparing the cytological abnormality incidence rates of immigrants from a high-risk area for cervical cancer with those of the source population. In **CHAPTER 3** we therefore compared cervical cytological abnormality incidence rates in Surinamese women living in Suriname and the incidence rates in the Surinamese immigrants living in the Netherlands. This scenario factors out endogenous differences, as the same ethnic population has been studied in two areas.

As previously discussed, immune surveillance for HPV associated lesions is performed by CTLs, which are activated by the antigen presentation of the human leukocyte antigen (HLA) class I molecules. In cervical cancer HLA class I aberrations are common. To determine the timing, frequency and mechanism of HLA class I downregulation in cervical carcinogenesis, we performed immunohistochemistry, loss of heterozygosity (LOH) analysis and fluorescent in situ hybridisation (FISH) on cervical carcinoma specimens and adjacent cervical intraepithelial neoplasia (CIN) lesions **(CHAPTER 4)**.

The frequently occurring HLA aberrations in cervical cancer are predominantly caused by extensive LOH at chromosome 6p21.3, partially in combination with mutations in β_2m or HLA class I genes. The significance of disturbed transporter function in cases with loss of HLA class I expression that could not be explained, needs to be explored. Low transporter associated with antigen processing (TAP) expression has previously been reported and associated with HLA class I downregulation in cervical carcinomas, but limited information exists about underlying mechanisms. In **CHAPTER 5** we investigated loss of TAP and HLA class I expression in invasive cervical carcinoma and adjacent precursor lesions, to determine the occurrence of TAP downregulation and its relation with HLA class I in cervical carcinogenesis. In addition, we examined possible causative mechanisms of the TAP downregulation by performing LOH and gene mutation analysis.

Up until now, it was the common opinion that, although precursor lesions may have multiple human papillomavirus (HPV) infections, invasive cervical carcinoma is a clonal process and therefore infected with only one HPV genotype. Recently, a technique better suited for detection of multiple HPV infections was developed. This permitted us to investigate the prevalence of multiple HPV infections in cervical cancer for a low-risk (Dutch) and a high-risk (Surinamese) population. Additionally, we examined whether cervical carcinomas with a multiple HPV infection are derived from one malignant clone infected with multiple HPV types or alternatively, whether multiple malignant clones developed to invasive carcinoma **(CHAPTER 6)**.

In **CHAPTER 7** several of the topics that are dealt with in this thesis are highlighted in a general discussion. Finally, the findings described in the aforementioned studies are summarised in English and Dutch **(CHAPTER 8 and 9)**.

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Chapter 2

Ethnic Patterns of Cytological Abnormalities in Cervical Smears in Suriname, a High-Risk Area for Cervical Cancer

Abstract

We determined the prevalence of cytological abnormalities in cervical smears of women attending the first organised screening programme in Suriname and to compare the prevalences in four Surinamese ethnicities with different cervical carcinoma incidence. Cervical scrapes were taken from women with four different ethnicities, i.e. Maroons, Amerindians, Javanese and Hindustani. Papanicolaou staining and cytological screening were performed on 807 cervical smears. Cervical cytological abnormalities were seen in 13.4%, of which 8.1% (62/764) with atypical changes, 2.6% (20/764) with mild and 2.6% (20/764) with moderate and severe dysplasia/CIS. The cytological abnormalities varied between the ethnicities, 42.1% (83/197) in the Maroons and 2.3% (4/176), 5.0% (9/183), and 3.0% (6/208) in the Javanese, Amerindians, and Hindustani, respectively. The high prevalence of moderate and severe dysplasia/CIS in all ethnicities correlates with the high cervical carcinoma incidence in Suriname. A significantly higher prevalence of mild abnormalities in the Maroons was observed, which did not reflect the relatively low cervical cancer incidence in this ethnicity. However, this can be explained by the possibility that these women have a different sexual lifestyle, leading to a higher prevalence of transient HPV infection.

Introduction

Cervical carcinoma is the second most common female cancer throughout the world with considerable differences in incidence rates. Worldwide, over 470,000 new cases are diagnosed yearly and it remains one of the leading causes of death from cancer among women[1-3]. The highest incidence rates are found in developing countries with age adjusted incidence rates of more than 20 per 100,000 women in South America[1-5].

The past decades both the cervical carcinoma incidence as well as the occurrence of the advanced FIGO stages[6] have decreased in developed countries. Screening programme in developed countries account for the majority of this decline in cervical carcinoma incidence and mortality rates although the impact has never been studied in randomised trials[5,7,8]. Decades after the implementation of screening programme, the cervical cancer incidence in the developed countries has decreased 30-60% to age standardised rates between 5.0 and 12.1[1,3-5,8]. Results of a recent case-control study indicated that the substantial decrease in cervical carcinoma incidence and mortality rates in Finland is mainly due to the organised mass screening[9].

Unfortunately, developing countries remain high-risk areas for cervical cancer. They account for 79% of the cervical cancer incidence worldwide and advanced FIGO stages are still of frequent occurrence in these countries[8]. Implementation of screening programmes in developing countries therefore seems an appropriate measure to decrease the high incidence.

Suriname is a high risk area for cervical carcinoma with an incidence of at least 26.7 per 100,000 women and a three- to six fold higher percentage of the advanced FIGO stages (IIB-IV)[10]. There are various ethnicities living in Suriname, which have a different cervical carcinoma incidence. We analysed cervical smears of four different Surinamese ethnicities to determine the prevalence of cytological abnormalities in cervical smears of Surinamese women attending the (preliminary) screening programme that started in 1997. In addition, we investigated whether the differences in cervical cancer incidence existing between the ethnicities was reflected in the proportions of cytological abnormalities.

Material and Methods

Study Population

The smears in this study were collected from Surinamese women attending the (preliminary) screening programme that was part of the bilateral medical care programme between Suriname and the Netherlands. Cervical smears from women of four different ethnicities, i.e. Maroons, Javanese, Amerindians, and Hindustani, were analysed for this study. In former studies about Suriname[10,11] Maroons were called “bush Negroes”. Recently

this ethnicity was renamed. The current name is used in this study. The smears were taken between April 1997 and December 2000 throughout the whole country. Two of our studied ethnicities (Javanese and Hindustani) reside predominantly in urban areas, i.e. Paramaribo, and two (Maroons and Amerindians) reside in the inlands of Suriname. The Maroons and the Hindustani have the lowest cervical carcinoma incidence, the Javanese and the Amerindians have the highest incidence in Suriname[10].

Cervical Smearing

A highly experienced and trained physician (A.G.) supervised and coordinated the practical part of the cervical screening programme. Under his guidance well-trained physicians (assistants) took cervical smears of all the Surinamese women. The majority of the smears were taken in one of the mobile medical units or at a medical clinic in Paramaribo.

A smear sample was taken and spread on two glass slides, fixed and stored at room temperature until use. For every woman a new, disposable cervix brush was used. One glass slide was included in the Surinamese screening programme, the other was shipped to Leiden for review.

Cytological Diagnosis

After shipping the material to Leiden, the Netherlands, standard Papanicolaou staining was performed on all samples for diagnostic purposes. The smears were reviewed by qualified cytotechnologists and a cytopathologist for adequacy, presence of inflammation, pathogens and cytological atypia. Cervical cytological abnormalities were graded using the KOPAC system, the official Dutch microscopical coding system[12,13]. A Pap score was given for communication with clinician and patient. Furthermore, special attention was paid to signs of viral infection, as well as other cervical infections. The term "(cervical) cytological abnormalities" was merely used for atypical or dysplastic changes and not for changes caused by cervical infections.

Statistical Analysis

Odds ratios (OR) and age adjusted ORs with a 95% confidence interval (CI) of cytological findings were calculated for the different ethnicities by using logistic regression. The Hindustani was used as reference ethnicity, because their cervical carcinoma incidence is the average of all ethnicities in Suriname. Both the odds for squamous atypia and higher, and for mild dysplasia and higher were estimated. The reason for this is that smears with squamous atypia can neither be classified normal nor dysplastic. Recent studies show the broad variation of follow-up cytological and histological diagnoses after a first diagnosis of squamous atypia or ASCUS, underlining the necessity of this transition group[14-17].

Results

The age distribution of the four studied ethnic groups is shown in **FIGURE 1**. The median age of the ethnicities was 34 for the Hindustani (mean 35.1 years), the Amerindians (mean 35.8 years), the Maroons (mean 38.1 years) and 34.5 for the Javanese (mean 36.4 years).

Cytopathological Diagnosis

TABLE 1 shows a comparison of four cytological classifications for cervical squamous cells that are frequently used in cytopathology. In the total group 5.3% (43/807) of the smears were unsuitable for cytological evaluation. Cervical cytological abnormalities were detected in 13.4% (102/764) of the assessable smears. Squamous atypia was seen in 8.1% (62/764), mild dysplasia in 2.6% (20/764) and moderate and severe dysplasia/CIS in 2.6% (20/764) of the smears. **TABLE 2** shows the high prevalence of squamous atypia in the

TABLE 1

Comparison of four cytological classifications for cervical squamous cells[27]

Classification			**Stage**			
Bethesda 2001	NILM	ASC-US ASC-H	(L)SIL	(H)SIL		Invasive carcinoma
CIN Nomenclature	Negative	Squamous Atypia	CIN I	CIN II	CIN III	Invasive carcinoma
Dysplasia Nomenclature	Negative	Squamous Atypia	Mild Dysplasia	Moderate Dysplasia	Severe Dysplasia/ CIS	Invasive carcinoma
Papanicolaou Classification	Pap I	Pap II	Pap IIIA*		Pap IIIB/IV	Pap V

*Pap IIIA consists of both mild and moderate dysplasia

TABLE 2

Prevalence of the Cytological Diagnoses in the Assessable Smears per Ethnicity

Ethnicity	**No. Tested**	**Normal Cytology (%)**	**Squamous Atypia (%)**	**Mild Dysplasia (%)**	**Moderate Dysplasia (%)**	**Severe Dysplasia/ CIS (%)**
Maroons	197	114 (58)	57 (29)	15 (7.6)	3 (1.5)	8 (4)
Javanese	176	172 (97.7)	1 (0.6)	3 (1.7)	0 (0)	0 (0)
Amerindians	183	174 (95)	2 (1)	0 (0)	2 (1)	5 (3)
Hindustani	208	202 (97)	2 (1)	2 (1)	2 (1)	0 (0)
All samples	764	662 (86.6)	62 (8.1)	20 (2.6)	7 (0.9)	13 (1.7)

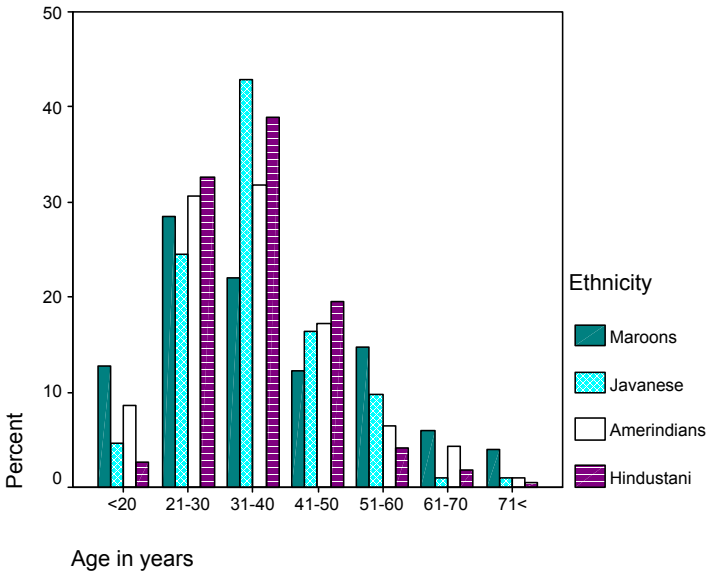

FIGURE 1
Prevalence of the Age Groups per Ethnicity

Maroons (29%) and the lower prevalence in the other ethnicities (1%). It also shows the significantly higher prevalence of mild dysplasia in the Maroons (7.6%) compared to the Hindustani, the Javanese and the Amerindians (1%, 1.7% and 0%). Moderate and severe dysplasia/CIS was observed in respectively 5.5%, 1%, 0% and 4% of the Maroons, the Hindustani, the Javanese and the Amerindians (**TABLE 2**).

The odds ratios and the age adjusted odds ratios of the cytological findings per ethnicity are shown in **TABLE 3**. The odds ratios for squamous atypia and higher is shown in **TABLE 3A**. The highest age adjusted odds ratio of 26.0 (CI 10.7-62.8) was seen in the Maroons. The odds ratios for mild dysplasia and higher are shown in **TABLE 3B**. In the Maroons an age adjusted odds ratio of 8.8 (CI 2.9-26.4) was seen and in the Amerindians an age adjusted odds ratio of 2.13 (CI 0.6-7.4).

Cervical Infections

Virally induced changes, like koilocytosis, were seen in 7.6% of the assessable smears. These changes were present in 2.9% of the Hindustani, 1.7% of the Javanese, 2.2% of the Amerindians and 22.8% of the Maroons (data not shown). In 7.5% of the smears evidence of a Trichomonas infection was seen. In the Hindustani, the Javanese and the Amerindians in 1.4%, 0.6%, and 2.2%, respectively, in de Maroons in 24.9% of the assessable smears. In the Maroons 22.8% of the smears with squamous atypia were infected with Trichomonas (**TABLE 4**).

TABLE 3

Odds Ratios for Cytological Abnormalities in Assessable Smears per Ethnicity

3A. Odds Ratios for Squamous Atypia and Higher

Ethnicity	No. Tested	Sq. Atypia < (%)	OR	95% CI	OR_{adj} [a]	95% CI_{adj} [a]
Maroons	197	83 (42)	24.51	10.37-57.90	25.97	10.74-62.81
Javanese	176	4 (2.3)	0.78	0.23-2.82	0.74	0.21-2.67
Amerindians	183	9 (5)	1.74	0.61-4.99	1.77	0.62-5.10
Hindustani	208	6 (3)	1.00	reference	1.00	reference

[a]Adjusted for age groups

3B. Odds Ratios for Mild Dysplasia and Higher

Ethnicity	No. Tested	Mild Dyspl. < (%)	OR	95% CI	OR_{adj} [a]	95% CI_{adj} [a]
Maroons	197	26 (13.1)	7.75	2.65-22.66	8.80	2.94-26.38
Javanese	176	3 (1.7)	0.88	0.20-4.01	0.85	0.19-3.86
Amerindians	183	7 (4)	2.03	0.58-7.04	2.13	0.61-7.44
Hindustani	208	4 (2)	1.00	reference	1.00	reference

[a]Adjusted for age groups

TABLE 4

Prevalence of Trichomonas Infection in the Assessable Smears per Ethnicity and Cytological Diagnosis

Ethnicity	No. Tested	Normal Cytology (%)	Squamous Atypia (%)	Mild Dysplasia (%)	Moder./severe Dysplasia/CIS (%)
Maroons	197	34/114 (29.8)	13/57 (22.8)	2/15 (13.3)	0 (0)
Javanese	176	1/172 (0.6)	0 (0)	0 (0)	0 (0)
Amerindians	183	4/174 (2.3)	0 (0)	0 (0)	0 (0)
Hindustani	208	3/202 (1.5)	0 (0)	0 (0)	0 (0)
All samples	764	42/662 (6.3)	13/62 (21.0)	2/20 (10.0)	0 (0)

Discussion

A high prevalence of moderate and severe dysplasia/CIS was found in this study of a high-risk population for cervical carcinoma. Cervical cytological abnormalities were detected in 13.4% of the assessable smears, of which 2.6% was moderate and severe dysplasia/CIS. In the smears of the Maroons significantly more cytological abnormalities

were detected. Remarkably, their cervical cancer incidence is among the lowest of the Surinamese ethnicities.

In the developed countries the cervical cancer incidence has decreased substantially decades after the implementation of screening programmes[1-5]. The programme in Finland is the model for organised programmes of screening by cervical cytology worldwide[18]. Between the implementation in 1963 and 1990 when the data was established, there has been an 80% decrease in age-adjusted incidence of, and mortality from, cervical cancer[19]. The detection rate of dysplasia in cervical smears in Finland was 0.7% in 1999[20]. In the Netherlands, which is also a low-risk country with a well organised screening programme since decades, moderate and severe dysplasia/CIS was detected in 0.6% in 2001[21].

Since cervical carcinoma is the most common cancer among women in developing countries, the World Health Organization (WHO) recommends the implementation of organised screening programme for cervical cancer in all high-risk areas[5]. In Suriname a nation wide screening programme was initiated in 1998. A high prevalence of cytologically abnormal smears was expected and our study does indeed show a high prevalence of especially moderate and severe dysplasia/CIS. This correlates with the high cervical cancer incidence in Suriname[10].

In Cameroon liquid-based and conventional cytology was compared. ASCUS/LSIL was detected in 10.1%, HSIL in 2.5% (liquid-based cytology)[22]. These prevalences are similar to the prevalences detected in our study. They are higher than the prevalences in the Netherlands[20,21,23]. They are also higher than the prevalences in Finland, another low-risk country, but the ASCUS prevalence is similar to that in Finland[24]. Women with dysplasia will be treated if histological analysis shows CIN and this prevents further carcinogenesis and therefore invasive carcinoma.

In the Maroons a much higher prevalence of squamous atypia and mild dysplasia was present, which could be caused by a possible higher HPV prevalence in this ethnicity. Indeed, HPV-suggesting abnormalities (mainly koilocytosis) were significantly higher among the Maroons (22.6%). This is a strikingly high number when compared with for instance similar data reported from the mass screening in Finland (around 1%)[25,26]. Koilocytosis is associated with clinically active viral infections, the vast majority of which are caused by low-risk HPV types. Koilocytosis becomes progressively more rare among infections caused by high-risk HPV types, which induce a transforming infection with progressive cytological atypia. The productive HPV infections are normally rapidly regressing. Our findings are most feasibly explained by the likelihood of a more active sexual lifestyle among the Maroons. This is manifested as the higher Trichomonas infection rate and the extremely high koilocytosis incidence. The latter relates to a high prevalence of low-risk HPV infections, which results in a high prevalence of predominantly mild abnormalities. As these represent transitory events, they do not necessarily relate to the cervical cancer incidence. The prevalence of moderate and severe dysplasia of the Maroons approached

that of the other ethnicities, which is more in accordance with their relatively low cervical carcinoma incidence. Remaining inconsistencies could be influenced by the fact that our interpretation of abnormalities is limited to cytology alone.

The prevalence rates we found could be influenced by an incomplete attendance and coverage of the population screening in Suriname. The coverage in Suriname for the females in the target age was approximately 50%. Successful screening programmes in developed countries cover at least 60% of the female population at risk, which is screened at regular intervals[8,20,21,23]. There was no significant difference in coverage between the various ethnicities in Suriname.

In conclusion, the high prevalence of moderate and severe dysplasia/CIS in all ethnicities correlates with the high cervical carcinoma incidence in Suriname. The higher prevalence of mild cervical cytological abnormalities in Maroons does not reflect their relatively low cervical cancer incidence. This is, however, likely to be due to a higher transient HPV infection prevalence, most feasibly explained by the likelihood of a more active sexual lifestyle.

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Chapter 3

Decreased Prevalence of Dysplasia in High-Risk Population Immigrants in a Low-Risk Area for Cervical Cancer

Abstract

Incidence rates of cervical cancer and its precursors vary considerably, with the highest rates found in developing countries. Differences are influenced by endogenous and exogenous factors. Comparing cytological abnormality incidence rates from a high-risk population in the original high-risk area with those of women from this high-risk population who have immigrated to a low-risk area could give insight in the significance of endogenous versus environmental factors. Smears collected from Surinamese women attending the Surinamese screening programme and smears collected from immigrant Surinamese women attending the Dutch screening programme were cytologically analysed using the Dutch microscopical coding system KOPAC. Statistical analysis was performed by using logistic regression to calculate (age-adjusted) odds ratios. The age-adjusted odds ratios of having dysplasia were higher for Surinamese women living in Suriname versus Surinamese immigrant women and increased with increasing P-scores: 0.77 (0.31-1.91) for borderline changes, 1.62 (0.58-4.57) for mild dysplasia and 3.20 (1.55-6.60) for moderate to severe dysplasia/neoplasia. We conclude that fewer cases with dysplasia are present in a high-risk population that has immigrated to a low-risk area for cervical cancer than in the high-risk population continuously living in a high-risk area. This finding emphasises the importance of environmental factors.

Introduction

Cervical carcinoma remains the second most common cancer among women worldwide, with over 470,000 new cases diagnosed yearly[1]. Incidence rates of cervical cancer and premalignant cervical lesions vary considerably and the highest incidence rates are found in developing countries[1-3]. The differences in incidence rates are influenced by exogenous and endogenous factors[4]. Important exogenous or environmental factors are human papillomavirus (HPV, the unifying risk factor for cervical cancer[5,6] and its precursors[7]), screening history and sexual lifestyle[4,8]. Endogenous factors consist of immunogenetic characteristics among others. Although these risk factors have been studied previously, the relative influence of endogenous and environmental factors on the differences in incidence rates between high- and low-risk areas remains unclear. It is possible to obtain insight in this matter by comparing the cytological abnormality incidence rates of immigrants from a high-risk area for cervical cancer with those of the source population.

Suriname is a high-risk area for cervical carcinoma with an incidence of at least 26.7 per 100,000 women and a three- to sixfold higher percentage of the advanced FIGO stages (IIB-IV) than found in low-risk areas[9]. Almost half of the Surinamese population lives in the Netherlands, as a result of Suriname being a former Dutch colony. The demographics of the Surinamese immigrants in the Netherlands and the source population in Suriname are similar[10,11], which provides us with a unique opportunity for research. The purpose of this study was to compare cervical cytological abnormality incidence rates in a high-risk population living in a high-risk area for cervical cancer and the incidence rates in members of the same high-risk population who have emigrated and are living in a low-risk area.

Material and Methods

Surinamese Study Population from the Surinamese Screening Programme

In Suriname the nationwide screening programme started as part of the bilateral medical care programme between Suriname and the Netherlands. It targets women aged between 20 and 55. For most Surinamese women this is their first smear taken ever, as this is the first screening programme implemented in Suriname. All smears are analysed by the Cytology Department of the Lobi Foundation and by the Department of Pathology, Academic Hospital, Paramaribo, Suriname. The response rate of the targeted women of the Surinamese screening programme in this period was estimated at 50%. Between 1997 and 2001 a random sample of the smears (n = 890, stratified by race) was also analysed by the Department of Pathology, Leiden University Medical Centre, Leiden, the Netherlands. For this study, the smears analysed in the Netherlands were used. Only smears from ethnic

groups who have immigrated to the Netherlands were included in the study population. The final number of smears from Suriname in this study was 686.

Surinamese Study Population from the Dutch Screening Programme

The regular Dutch screening programme targets women in the Netherlands between 30 and 60 years of age. Among these women are Dutch citizens and legal immigrants. The smears of all women attending the screening programme in the Western region of the Netherlands between 1997 and 2001 were collected. From these smears, those of women born in Suriname were selected, and numbered 7613 in total. All immigrant Surinamese women in this study are, therefore, first-generation immigrants. The smears used in this study were the first smears taken of these women. The Surinamese immigrants are from all social levels of the Surinamese population[10] and are comparable with the source population in terms of socio-economic status and demographic characteristics[11]. The response rate of immigrant Surinamese women in this period was 58% (Dr M.E. Boon, SBBW, the Netherlands).

Cervical Smearing

The majority of the smears in Suriname were taken in one of the mobile medical units or at a medical clinic in Paramaribo. A smear sample was taken and spread on two glass slides, fixed and stored at room temperature until use. For every woman a new, disposable cervix brush was used. One glass slide was included in the Surinamese screening programme, the other was shipped to Leiden, the Netherlands for review. The smears in the Dutch screening programme were mostly taken at general physician practices throughout the western region of the Netherlands.

Cytological Diagnosis with KOPAC

The smears from Suriname were shipped from Suriname to Leiden. On all Surinamese smears, both from the Surinamese and the Dutch screening programme, standard Papanicolaou staining was performed for diagnostic purposes. The smears were reviewed by different laboratories in the western part of the Netherlands using the same protocol for cytological analysis. Cytological findings were coded using the KOPAC system, since the 1980s the official Dutch microscopical coding system for cytological analysis of cervical smears. Studies comparing different laboratories using the KOPAC system revealed no significant inter-laboratory differences in cytological scores (data not shown).

Smears are given a P-score for normal squamous epithelium (P1), borderline changes (P2-3) and (pre)neoplastic changes in the squamous epithelial cells, varying from P4 (mild dysplasia) to P9 (invasive squamous cell carcinoma) (**TABLE 1**). Inflammation is coded with "O" and consists of different codes for the variety of inflammation types. Koilocytosis, a cavity around the nucleus, was coded as "O1" for cells with and without abnormalities.

TABLE 1
KOPAC, the official Dutch microscopical coding system for cytological analysis of cervical smears. Description and translation of codes for normal squamous epithelial cells and (pre)neoplastic changes, the "P-codes"

KOPAC Code	Description	Bethesda
P1	Normal	Normal
P2-3	Borderline Changes	ASCUS
P4	Mild Dysplasia	(L)SIL
P5	Moderate Dysplasia	(H)SIL
P6	Severe Dysplasia	(H)SIL
P7	Carcinoma in Situ	(H)SIL
P8	Micro invasive Carcinoma	Carcinoma
P9	Squamous Cell Carcinoma	Carcinoma

Statistical Analysis

Odds ratios (OR) and age-adjusted ORs with 95% confidence intervals (CIs) of the (pre) neoplasia P-scores were calculated for the Surinamese screening population from Suriname versus the immigrant Surinamese screening population from the Netherlands by using logistic regression. The smears scored P1 (normal squamous epithelial cells) were used as a reference group.

Results

The median age for Surinamese women attending the Surinamese screening programme (SuS) was 34 (mean 35.5) years and the range was 14 to 75. For immigrant Surinamese women attending the Dutch screening programme (SuN), the median age was 40 (mean 41) years with a range of 30 to 63. This difference is due to the fact that the screening programme in Suriname starts at an earlier age (20) than does the programme in the Netherlands (30).

Smears were unsuitable for cytological analysis in 46/686 (6.7%) of the SuS and in 73/7613 (1.0%) of the SuN. The observed prevalence of (pre)neoplastic changes in the SuS was higher for mild (P4) and moderate to severe dysplasia/neoplasia (P5-9) compared to the SuN (**TABLE 2**).

The age-adjusted odds ratios for SuS versus SuN increased with increasing P-scores up to OR = 3.2 (CI 1.55-6.60) for P5-9. The odds ratios and the age-adjusted odds ratios of the different (pre)neoplastic stages are shown in **TABLE 2**.

The abnormal smear prevalence (P≠1) per age group among the SuS was 7/196 (3.6%) for < 30, 12/248 (4.8%) for 30-39, 3/123 (2.4%) for 40-49 and 3/73 (4.1%) for ≥ 50 years.

TABLE 2

P-Scores, odds ratios and age-adjusted odds ratios of (pre)neoplastic changes (P2-9) for Surinamese women living in Suriname

(Pre) neoplastic changes	SuS[1] (n = 640) N (%)	SuN[2] (n = 7540) N (%)	OR^{3}_{crude}	95% CI^{4}_{crude}	OR_{adj}[5]	95% CI_{adj}[5]
P1	615 (96.1)	7340 (97.4)	1.00	reference	1.00	reference
P2-3	6 (0.9)	111 (1.5)	0.65	0.28-1.47	0.77	0.31-1.91
P4	9 (1.4)	40 (0.5)	2.69	1.30-5.56	1.62	0.58-4.57
P5-9	10 (1.6)	49 (0.6)	2.44	1.23-4.83	3.20	1.55-6.60

[1]Smears from Surinamese women living in Suriname

[2]Smears from Surinamese women living in the Netherlands

[3]Odds Ratio

[4]Confidence Interval

[5]Adjusted for age groups

TABLE 3

Correlation of normal squamous epithelial cells (P1) and (pre)neoplastic changes (P2-9) with koilocytosis (O1)

(Pre) neoplastic changes	No. SuS[1]O1 N (%)	No. SuN[2]O1 N (%)
P1	1/615 (0.2)	1/7340 (0)
P2-3	2/6 (33.3)	13/111 (11.7)
P4	8/9 (88.9)	3/40 (7.5)
P5-9	7/10 (70.0)	1/49 (2.0)
All	18/640 (2.8)	18/7540 (0.2)

[1]SuS = Smears from Surinamese women living in Suriname

[2]SuN = Smears from Surinamese women living in the Netherlands

For the SuN it was 86/3011 (2.9%) for 30-39, 89/3155 (2.8%) for 40-49 and 25/1374 (1.8%) for ≥ 50 years.

Adjusting odds ratios after exclusion of the SuS aged < 30 did not alter our results.

Koilocytosis occurred over ten times more frequently in the SuS (18/640 = 2.8%) than in the SuN (18/7540 = 0.2%). Koilocytosis (O1) was only observed in a minority of both study groups, but a correlation between koilocytosis and (pre)neoplastic changes could nevertheless be found. In both populations, 17/18 cases of koilocytosis observed occurred in smears with borderline changes (P2-3) and (pre)neoplastic changes (P4-9) (**TABLE 3**). Smears negative for inflammatory changes were diagnosed in 5267/7540 (69.9%) and in 224/640 (35.0%) individuals, respectively.

Discussion

This study shows that fewer dysplasias are present in a high-risk population that has immigrated into a low-risk area for cervical cancer than in the high-risk population continuously living in the high-risk area. This emphasises the significance of environmental factors for differences in the geographical incidence of cervical cancer and its precursors.

Migrant populations are a non-random, self-selected sample of the population of their country of origin, which could give rise to a selection bias when comparing the two. The effect of selection bias is reduced if comparisons can be made between similar groups[12]. The Surinamese population in the Netherlands is demographically comparable with that in Suriname[10,11]. Furthermore, it is unlikely that cervical (pre)neoplastic changes would positively or negatively influence (Surinamese) women's migration behaviour. The vast majority of the Surinamese immigrants lives in urban areas of the Netherlands, which is similar to the situation in Suriname where over 90% of the Surinamese population resides in greater Paramaribo.

We found that the age-adjusted odds for the SuS of developing mild (P4) and moderate to severe dysplasia/neoplasia (P5-9) are higher and increase with (pre)neoplasia grade compared to the SuN (**TABLE 2**). One of the possible causes could be that HPV infection, which precedes cervical dysplasia[13,14], is more common in high-risk areas for cervical cancer[3,15-17], including Suriname[18]. This could be associated with the sexual lifestyle encouraged by the Surinamese culture, as was established in several populations[8,14,19]. Furthermore, a higher viral load and different HPV type variants in HPV-positive women in a high-risk area could cause an increased risk of cervical cytological abnormalities, as suggested recently[20-22].

In addition, the fact that the organised screening programme for cervical cancer has only recently started in Suriname could account for the higher outcome in the SuS[22]. This is supported by the fact that the majority of the decline in cervical carcinoma incidence rates in developed countries is attributed to the implementation of organised screening programmes[2]. The protective effect of previous screening, independent of HPV, has also been established[8]. One should keep in mind that, in both groups, the (pre)neoplastic changes were observed in low numbers which may unduly influence the odds ratios.

The prevalence of dysplasia in Dutch women is 0.4 % for both mild and moderate to severe dysplasia, which was established in the same region and time frame as the present study[23]. A recent study in which cervical smears of multiple immigrant populations in the Netherlands were investigated, revealed a somewhat higher relative risk for mild to severe dysplasia/neoplasia in the immigrant Surinamese smears compared to smears from the Dutch population[24]. Although the prevalence of dysplasia in Surinamese immigrant women in the Netherlands has decreased, it has not completely diminished to the level of the Dutch women in the Netherlands. This could indicate that they have not

yet completely adjusted their lifestyle to their new environment, especially given that all immigrant Surinamese women in this study are first-generation immigrants. However, we cannot exclude endogenous factors as a possible cause. When feasible, a similar study on the offspring of these immigrants (second generation) could investigate whether the prevalence of dysplasia has further decreased to that of the native Dutch population.

In our study we also found a positive correlation between cytological abnormalities (P2-9) and koilocytosis (O1), which is stronger for the SuS (**TABLE 3**). This confirms a recent study of cervical smears from the Dutch screening programme[24] and is also supported by several studies on koilocytosis and cervical carcinogenesis[25,26]. Koilocytosis is considered to be virally induced and has been correlated with HPV[27-29]. The fact that koilocytosis occurred over ten times more frequently in the SuS suggests a higher prevalence of HPV in this population, which is supported by a recent study[18]. It could furthermore be caused by higher viral load or different HPV type variants, as a result of the previously proposed differences in exposure between the high-risk and the low-risk area studied. Koilocytosis can be unequivocally detected in cervical smears and might be an effect of the active stage of HPV infection[24]. It has been positively correlated to promiscuity, which could explain why a greater number of active HPV infections were observed in the SuS[30]. In the Dutch population only 4% of the smears with moderate to severe dysplasia also contain koilocytosis[24], which may indicate that the HPV infection becomes less active in high-grade lesions. An even lower frequency was observed in the SuN (2%). This is not the case for the SuS where as many as 7/10 of the smears with moderate to severe dysplasia showed koilocytosis, possibly caused by (repeated) reinfection with HPV.

In summary, we have shown that fewer cervical cytological abnormalities are present in a high-risk population that has immigrated to a low-risk area for cervical cancer than in the high-risk population continuously living in a high-risk area. This scenario factors out endogenous differences, as the same ethnic population has been studied in two areas. The findings in this study emphasise the significance of environmental factors, such as HPV exposure. In addition, the presence of an organised screening programme is important. However, endogenous factors and maintained sexual lifestyle should also be considered to be of influence, with the latter related to the fact that the immigrants in this study are first-generation immigrants. The higher frequency of koilocytosis in the women still living in Suriname deserves further study with regard to repeated reinfection with HPV as a cause of the more frequently observed active stage of the infection.

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Chapter 4

Frequent HLA Class I Loss is an Early Event in Cervical Carcinogenesis

Abstract

Loss at chromosome 6p21.3, the human leukocyte antigen (HLA) region, is the main cause of HLA downregulation, occurring in the majority of invasive cervical carcinomas. To identify the stage of tumour development at which HLA class I aberrations occur, we selected 12 patients with cervical carcinoma and adjacent cervical intraepithelial neoplasia (CIN). We investigated HLA class I and β_2-microglobulin expression by immunohistochemistry in tumour and adjacent CIN. Loss of heterozygosity (LOH) was studied using microsatellite markers covering the HLA region. Fluorescence in situ hybridisation (FISH) with HLA class I probes was performed to investigate the mechanism of HLA loss. Immunohistochemistry showed absent or weak HLA class I expression in 11/12 cases. In 10 of these 11 cases downregulation occurred in both tumour and CIN. Only in one case did the concomitant CIN lesion show normal expression. In 9/12 cases LOH was present for at least one marker in both tumour and CIN, one case showed only LOH in the CIN lesion and one case showed retention of heterozygosity (ROH) for all markers in both tumour and CIN. We conclude that HLA class I aberrations occur early and frequently in cervical carcinogenesis. This might allow premalignant CIN lesions to escape immune surveillance and progress to invasive cancer.

Introduction

Cervical carcinoma is the second most common female cancer worldwide[1-3] and human papillomavirus (HPV) is its most important aetiological factor[4-7]. Immunological surveillance of HPV-associated lesions is performed by T-cells, which are activated when foreign (antigenic) proteins are presented to the T-cell receptor by human leukocyte antigen (HLA) class I proteins. HLA class I molecules are expressed on the cell surface and consist of a polymorphic heavy α chain, encoded by the HLA class I genes HLA-A, -B and -C on chromosome 6p21.3, in non-covalent association with the light β chain, encoded by the β_2-microglobulin (β_2m) gene on chromosome 15q21. This association is a prerequisite for the stability of the HLA class I molecule[8].

Loss of HLA surface expression occurs in various solid tumours and tumour cell lines[9,10] and might result in escape from cytotoxic T-cell attack. It occurs frequently in cervical carcinoma and is predominantly caused by genetic aberrations at chromosome 6p21.3. Koopman *et al.* showed that 50% of multiple HLA allele loss is caused by LOH in the HLA region[11], which is frequently detected in cervical cancer[12-16].

Invasive cervical carcinoma is preceded by three stages of cervical intraepithelial neoplasia (CIN). Several studies have shown that the majority of the untreated mild dysplasias regress to normal cytology and only a small proportion of the CIN lesions eventually progress to invasive carcinoma[17-19]. It is conceivable that the progressive CIN lesions have escaped immune surveillance. Several studies have recently investigated these precursor lesions for losses at 6p21.3, without distinguishing between progressing and regressing CIN lesions[20,21].

We selected patients with cervical carcinoma and adjacent CIN lesions to investigate how early and frequently HLA aberrations occur in cervical carcinogenesis. By choosing adjacent CIN, we were able to come as close as possible to selecting only progressive CIN lesions. By including these precursor lesions, we could add to the current knowledge of HLA aberrations during the development of cervical carcinoma.

Material and Methods

Tissue Samples

Since 1989, Surinamese women with cervical carcinoma have come to the Leiden University Medical Centre (LUMC) at Leiden, the Netherlands, to have a Wertheim operation, which is a radical hysterectomy. All the resected tumour tissue is stored in the tissue archive of the Pathology Department of the LUMC. From this group we selected the cases with invasive cervical carcinoma and an adjacent high-grade CIN lesion (CIN III) by reviewing the haematoxylin-eosin-stained slides. We found 15 cases with cervical cancer and an

adjacent CIN lesion, operated between 1989 and 1999, of which 12 had sufficient tissue to investigate. From these 12, tissue blocks containing formalin-fixed paraffin-embedded normal tissue and tumour tissue with adjacent CIN lesion were selected. To study intra-tumour heterogeneity we selected multiple tumour loci per case when possible. In nine cases two tumour sites and in one case three tumour sites were investigated.

All samples were positive for a high-risk HPV genotype, except for S70 which is HPV negative.

Immunohistochemistry

Immunohistochemistry was performed on freshly cut, 3-μm thick buffered, paraffin-embedded tissue sections according to standard procedures[22]. Slides were incubated overnight with mouse monoclonal antibodies (mAbs) suitable for paraffin sections: HCA2 and HC10 (Dr J. Neefjes, NKI, Amsterdam, the Netherlands) and the primary rabbit polyclonal anti-β_2m (A 072; DAKO, Copenhagen, Denmark). HCA2 recognises a determinant expressed on β_2m-free HLA-A (excluding HLA-A24), HLA-B7301 and HLA-G heavy chains[23,24]. The mAb HC10 recognises a determinant on all β_2m-free HLA-B and HLA-C heavy chains, as well as on β_2m-free HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32 and HLA-A33 heavy chains[23,25]. Immunodetection was performed as previously described[10].

In each tumour, stromal cells including lymphocytes served as a positive control for HLA class I expression. Tumour cells were only scored negative if no staining was present as compared to a strong staining of internal control cells. If some staining was present but reactive cells stained much stronger, tumour cells were scored as weakly positive.

Microdissection, DNA Extraction and Quantification

DNA was extracted as previously described, with minor adjustments[26]. Paraffin-embedded (normal and tumour/CIN) tissues of the 12 cases were cut in 10 μm sections and stained with haematoxylin. Before the normal dehydration steps, the staining procedure was interrupted to use the slides for microdissection. CIN lesions were microdissected using a needle under direct light-microscopic visualisation. Areas containing over 70% tumour cells, as well as normal control tissue were obtained using the same procedure and all tissue was transferred to sterile microcentrifuge tubes and incubated for 12 hours in 186 μl of PK1 buffer (10 mM Tris pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20, 0.01% gelatine), 5% Chelex (Chelex 100; Bio-Rad Laboratories, Hercules, CA) and 10 μl of a 10 μg/μl solution of proteinase K, at 56°C. This was followed by incubation at 100°C for 5 minutes to inactivate the proteinase K. After 5 minutes of centrifugation at full speed (16,060 x g) the supernatant was transferred to new sterile microcentrifuge tubes. The DNA content was quantified using Picogreen double-stranded DNA (dsDNA) quantification reagent (Molecular Probes Europe BV, Leiden, the Netherlands), an ultra-sensitive fluorescent nucleic acid stain for quantifying dsDNA in solution, according to the

manufacturer's instructions. Instead of the prescribed 10 mM Tris (pH 7.6), 0.1 mM EDTA dilution buffer, PK1 buffer was used, in accordance with the DNA isolation method.

LOH Analysis

DNA from microdissected normal, CIN and tumour material from all 12 cervical carcinoma cases was analysed for LOH on chromosome 6 by polymerase chain reaction (PCR) amplification using seven highly informative di-, tri- and tetranucleotide microsatellite markers[27,28]. These are listed in **TABLE 1**.

Standard PCR amplifications were performed according to a protocol previously described[22,29], with some adjustments. To circumvent PCR artefacts[30], 10 ng/µl purified template DNA was used in a 12 µl reaction volume containing 6 pmol of each primer, 2 mM dNTPs, 0.1 mg/ml BSA, Taq polymerase buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) gelatine, 0.1% Triton) and 1.0 unit AmpliTaq Gold polymerase (Perkin Elmer, Applied Biosystems Inc., Foster City, CA, USA). Either the forward or reverse PCR primer was fluorescently labelled with FAM or TET, respectively. Samples were denatured for 5 minutes at 96°C and amplified for 33 cycles consisting of 1 minute of denaturation at 94°C, 2 minutes of primer annealing at 55°C and 1 minute of elongation at 72°C, followed by a final extension step of 6 minutes at 72°C. For each primer set, PCR products of tumour DNA and normal DNA were mixed 1:1 and red-coloured GENE-SCAN-500 ROX (Perkin Elmer Cetus, Norwalk, CT, USA) was added as an internal DNA size marker. After denaturation and electrophoresis the PCR products were visualised as peaks and analysed

TABLE 1

Microsatellite markers, with primer sequence and locations [27,28]

Locus	Map Position	Forward and Reverse Primer Sequences Forward
D6S89	6p22.3	CAAGGGAATAGGTTAAGATTGCCA
		CATGAGAAGGCCCAGCTTGC
D6S105	6p22.1-6p21.3	GCCCTATAAAATCCTAATTAAC
		GAAGGAGAATTGTAATTCCG
MOGc	6p21.3	GAAATGTAGAATAAAGGAGA
		GATAAAGGGGAACTACTACA
D6S265	6p21.3	ACGTTCGTACCCATTAACCT
		ATCGAGGTAAACAGCAGAAA
C143	6p21.3	AGCCTGGGTGACAGAACAAG
		TGGATTAACCTGGAGACTCCTT
TNFa	6p21.3	GCCTCTAGATTTCATCCAGCCACA
		CCTCTCTCCCCTGCAACACACA
D6S1666	6p21.3	CTGAGTTGGGCAGCATTTG
		ACCCAGCATTTTGGAGTTG

on an ABI 310 automatic sequencer. GeneScan Analysis Software (Perkin Elmer, Applied Biosystems Inc., Foster City, CA, USA) was used to quantify each peak in terms of size (base pairs), peak area and height. Each PCR was performed at least twice.

The thresholds for retention of heterozygosity (ROH), "grey area" (allelic imbalance) and LOH were applied as previously empirically determined[31]. These were 0.76-1.3 (ROH), 0.58-0.75 & 1.31-1.69 (allelic imbalance) and 1.7< (LOH).

Interphase FISH analysis on Isolated Nuclei

To study the mechanism of HLA aberrations we performed interphase fluorescence in situ hybridisation (FISH) analysis with HLA-A and -B/C probes. In seven cases, nuclei were isolated from formalin-fixed, paraffin-embedded material as previously described[32]. The suspension was applied to microscope slides as described for interphase FISH on frozen material[33]. The slides were air-dried and used for hybridisation. All probes used were obtained and labelled as previously described[10]. The α-satellite centromeric 6-probe (D6Z1, Oncor, Gaithersburg, MD) was biotin-16-dUTP-labelled by nick translation (Roche, Basel, Switzerland). Cosmid c109K2118, derived from the ICRF flow-sorted chromosome 6 library, was obtained from the Resource Centre/Primary Database of the German Human Genome Project (Berlin, Germany). PAC238M10 was isolated from the RCPI-1 Human PAC Library of the Roswell Park Cancer Institute (obtained by Dr J. den Dunnen, Genome Technology Centre, LUMC, Leiden, the Netherlands) using an HLA-C probe. Cosmid and PAC probes were digoxigenin-12-dUTP-labelled by nick translation. Hybridisation was performed as previously described[10,33]. Hybridisation mixture (5 μl) that contained 3 ng/μl of the centromere 6 probe combined with 3 ng/μl of the cosmid or PAC probe, 1.5 μg human Cot-1 DNA and 3 μl hybridisation mix (50% formamide, 10% dextran sulphate, 50 mM sodium phosphate (pH 7.0), 2 x sodium chloride/sodium citrate [SSC]) was applied to the slides. After denaturation for 8 minutes at 80°C, nuclei were hybridised overnight at 37°C in a moist chamber. Immunodetection was performed as previously described[33]. Slides were analysed with a Leica DM-RXA fluorescence microscope (Leica, Wetzlar, Germany). Tonsils of healthy individuals were used as controls. The cut-off level for homozygous and hemizygous deletions was set as described previously[32].

Results

Loss of HLA class I expression detected in paraffin cervical tumour sections

Tissue sections with both tumour and CIN tissue were stained for β_2m, HLA-A and HLA-B/C expression (**FIGURE 1**). Using the available antibodies for use on paraffin sections, only the loss of both of the A- or B/C-alleles will result in a negative score. In S66 no expression of HLA-A, in S8 and S38 no expression of HLA-B/C and in S41 no expression

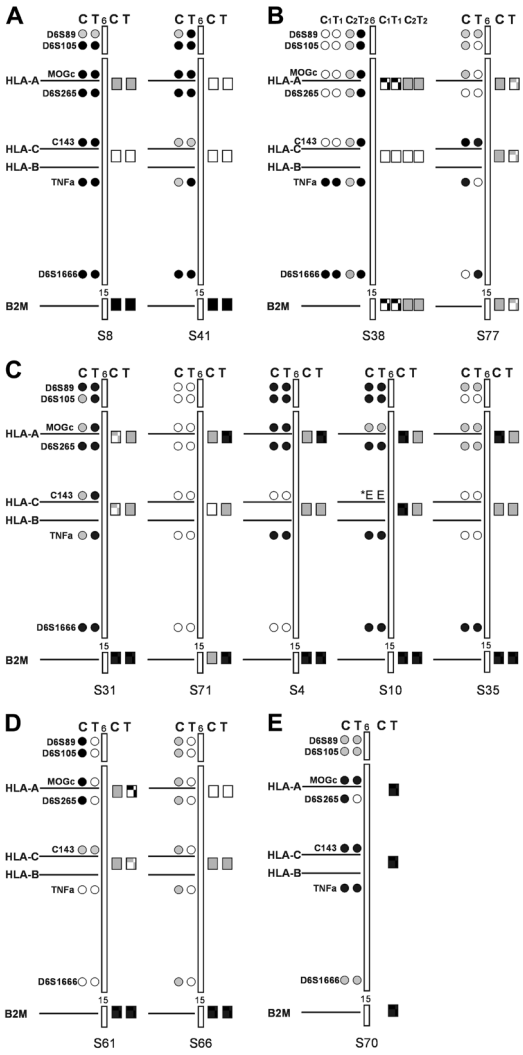

FIGURE 1

Analysed LOH data together with immunohistochemistry results. LOH results for seven markers on chromosome 6p for CIN (C) and tumour (T) are presented on the left side. ○ Retention of heterozygosity (ROH); ◍ Non-informative; ● Loss of heterozygosity (LOH). Immunohistochemistry results are presented on the right for the HLA proteins and for the β_2m protein (encoded on chromosome 15). □ Normal expression; ▨ Weak expression; ■ Absent expression; ▩ Heterogeneous: weak and normal expression; ▣ Heterogeneous: absent and normal expression. The cases are grouped with regard to the results obtained for the tumour samples studied. (**A**) No expression of one or both HLA class I molecules in combination with LOH on 6p21.3. (**B**) Downregulation of β_2m combined with ROH at 6p21.3. (**C**) Weak expression of HLA-A and/or HLA-B/C. (**D**) Lack of HLA class I expression combined with ROH and normal β_2m expression. (**E**) Normal HLA class I expression and LOH.

of both HLA molecules was seen in the invasive tumour tissue and adjacent CIN. Heterogeneous loss of expression with the two HLA class I antibodies was seen in three cases (S38, S77, S61), of which one (S38) had similar results in tumour and CIN. In four other cases homogenous or heterogeneous loss of expression was seen in the CIN lesions with one or both HLA class I mAbs, but weak expression was seen in the adjacent tumour tissue. Absent or very low β_2m expression provided an explanation for the absent or low expression of HLA-A and HLA-B/C in both tumour and CIN in two cases (S38, S77) and in the CIN lesion alone in one case (S71).

LOH Analysis in Tumour and Adjacent CIN Cases

We performed LOH analysis on 12 invasive cervical carcinomas with adjacent CIN III lesions using seven markers on chromosome 6p. Multiple tumour loci of the invasive tumour were studied, but in all patients except for one (S38) the results were similar. Only for this patient are the data from both tumour loci shown. An overview of the results is depicted in **FIGURE 1**.

In 9 of 12 cases at least one of the markers showed LOH in the invasive tumour and CIN lesion. In five of these nine cases, all markers showed LOH in both tumour and CIN lesion. In four of the nine cases, discrepancies between the results obtained for CIN lesion and tumour were seen (**FIGURE 1**). In S38, the LOH pattern of the accompanying CIN lesion differed only for D6S1666, a marker that showed ROH in the tumour. In S61, the tumour showed ROH for all markers, whereas the CIN lesion showed LOH for markers D6S89, D6S105, MOGc and D6S265. In S70, marker D6S265 showed ROH in the tumour and LOH in the CIN lesion. In S77, most markers showed ROH in the tumour and CIN, but at TNF-a LOH was detected only for CIN.

In S10, all markers showed LOH in both the tumour and CIN lesion, except for C143, located in the HLA-E – HLA-C region. This marker showed homozygosity in the normal sample, but two alleles of different molecular weights in both the tumour and CIN lesions. This phenomenon is termed elevated microsatellite instability at selected tetranucleotide repeats (EMAST) and has been described in other human cancers as a novel form of microsatellite instability (MSI)[34-36].

Interphase FISH Results

Interphase FISH analysis was performed on nuclei isolated from paraffin-embedded material of different tumour and CIN localisations from eight cases: S10, S16, S38, S41, S61 (C+T), S70, S71 and S77. HLA class I-specific clones (HLA-A and HLA-B/C) in combination with a probe for centromere 6 were applied. No homozygous or heterozygous deletions were found. Most cases showed nuclei with three and nuclei with four copies of chromosome 6. In all cases, nuclei with three copies were detected. The highest percentages were found in S10 (28%), S16 (31%) and S41 (59%). In all but two cases (S16 and S71), nuclei

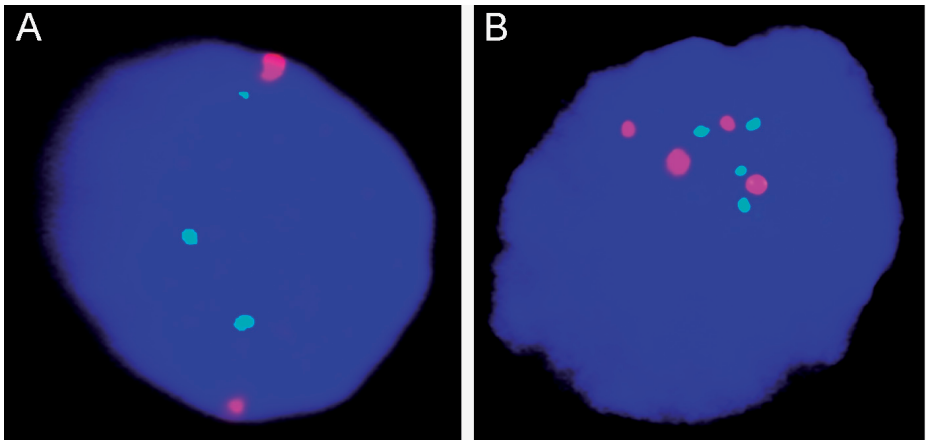

FIGURE 2

Examples of the FISH results. (**A**) In case S77, FISH showed two centromeric signals and three signals of the whole HLA class I region in 16% of the nuclei, suggesting a possible duplication and translocation. (**B**) Example of aneusomy 6, found in most of the cases.

with four copies of chromosome 6 were seen, with the highest percentage in S38 (28%). In one case (S77) FISH revealed two centromeric signals and three signals for the whole HLA class I region in 16% of the nuclei, suggesting a possible duplication and translocation (**FIGURE 2**).

Combined Allelic Imbalance and Immunohistochemistry Results

The analysed immunohistochemistry results together with the LOH data are presented in **FIGURE 1**. The cases are grouped with regard to the results obtained for the tumour samples studied. Because the majority of the results obtained by the different techniques used were in concordance, only the results whose interpretation is more complicated are reported below.

In S38, where CIN and tumour site 1 (C1+T1) showed ROH but site 2 showed LOH, the immunohistochemistry data also differed somewhat: in site 1 the expression was heterogeneous with both alleles absent from some of the cells, whereas site 2 had weakly expressed HLA-A in both the tumour and CIN. β_2m was weakly expressed in both tumour sites. In group **D**, lack of HLA class I expression in combination with ROH and normal β_2m expression was found in S61 (T) and in S66 (C+T). Remarkably, S61, with ROH in the tumour and LOH in the CIN, displayed (heterogeneous) negative expression of the HLA molecules in the tumour and weak expression in the CIN. Only in S70 was normal HLA class I expression seen despite LOH (group **E**).

Discussion

To investigate the timing, frequency and mechanism of HLA class I downregulation in cervical carcinogenesis, we performed immunohistochemistry, LOH analysis and FISH on cervical carcinoma specimens and adjacent CIN lesions. Including the precursor lesions in our study permitted us to add to the current knowledge of HLA aberrations in invasive cervical carcinoma[11]. The present study demonstrates that HLA class I downregulation occurs frequently and early in cervical carcinogenesis.

In cervical cancer LOH at chromosome 6p21.3, which occurs at high frequencies[12-16], represents an important and common mechanism by which HLA genes and their products are abolished[11]. This remains unclear in cervical precursor lesions. By choosing CIN III lesions that are adjacent to invasive cervical carcinoma, we were able to come as close as possible to selecting only CIN lesions that were progressing to invasive carcinoma. Nonetheless, we cannot exclude the possibility that multiple CIN lesions are present in the same patient. Thus, the CIN lesion adjacent to the invasive carcinoma could originate from a clonal process different from that of the invasive carcinoma.

The absence of HLA class I expression was explained by β_2m loss in two samples. This did not apply, however, to all HLA class I downregulation observed. Complex genetic changes involving various loci could be an alternative explanation. The weak but not absent expression of HLA class I that was observed in several samples suggests a genetic aberration in only one HLA allele, at 6p21.3. In most patients, CIN and invasive tumour samples provided similar results, supporting the hypothesis that both are from the same clonal process and demonstrating that HLA loss is an early event in cervical carcinogenesis. The specificity of antibodies available for immunohistochemistry on paraffin-embedded tissue is limited[23-25,37] and this might have led to an understatement of HLA downregulation in the present study.

Bontkes et al. reported loss of HLA class I expression in CIN lesions progressing from low to high grade, supporting the results in our study[38]. Several studies performed on solitary CIN lesions whose connection with invasive carcinoma was unknown did not find any HLA class I downregulation[39,40]. Other studies detected LOH at chromosome 6p21.3 in 25-75% of low and high grade CIN lesions[20,21]. We found even more LOH in our group, which could reflect the fact that it consisted of progressing CIN lesions.

Tumour heterogeneity occurs in cervical carcinoma[11] and can obviously be represented in different stages of carcinogenesis, explaining the rare differences observed between the results from tumour and adjacent CIN in the present study. When feasible, we selected multiple tumour loci per case (results not shown) to account for possible allelic imbalance variation caused by different tumour fractions, that is, diploid and aneuploid fractions. In one case, different tumour loci from the same cervical carcinoma had different LOH results and another case showed ROH for all markers in the tumour, but LOH in the CIN

lesion. This could implicate different clonal origins. In addition, in three of our cases some markers showed LOH in the CIN lesion but not in the invasive cancer. This could be due to the fact that the tumour tissue is frequently surrounded and infiltrated by lymphocytes that can contaminate the sample, whereas the CIN lesions are better separated from the stroma. Another possibility is that only HLA negative CIN lesions survived T-cell attack activated by the tumour's presence.

Several explanations exist for the multiple HLA aberrations we found in most of the tumour samples and the adjacent CIN lesions. The failure to express HLA class I could result from LOH at 6p21.3 in combination with a locus-restricted event in this area. Such an event might be a point mutation, small deletion, methylation, chromosome loss or large deletion. We found no homozygous or heterozygous deletions with FISH using cosmid and BAC probes covering the HLA-A and HLA-B/C region. We did find aneusomy 6 in most cases, which is in concordance with the study by Koopman *et al*.[11]. In one case we found a possible duplication and translocation of the HLA class I region (**FIGURE 2A**). Such a triplication should lead to LOH, but ROH was detected even in sorted cells and was probably caused by tumour heterogeneity.

We conclude that HLA class I aberrations occur not only frequently[11], but also early in cervical carcinogenesis. This phenomenon might allow the premalignant CIN lesion to escape immune surveillance and progress to invasive cancer.

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Chapter 5

Expression and Genetic Analysis of Transporter Associated with Antigen Processing in Cervical Carcinoma

Abstract

Transporter associated with antigen processing (TAP) loss causes human leukocyte antigen (HLA) class I downregulation which is frequently found in cervical carcinomas and their precursors. HLA class I molecules activate T-cells by antigen presentation and are therefore essential for immunological surveillance. To add to the hitherto limited knowledge of molecular mechanisms underlying TAP loss we investigated TAP expression, loss of heterozygosity (LOH) and possible TAP mutations. Twenty-three cervical carcinomas and adjacent precursor lesions were stained with HLA-A-, HLA-B/C-, β_2-microglobulin-, TAP1- and TAP2- antibodies.

In order to separate tumour and non-tumour cells, cervical carcinoma samples were sorted by flow-cytometry and were subsequently analysed for LOH with 3 markers in the TAP region on chromosome 6p21.3. Mutation analysis of the complete TAP1 gene was performed. Aberrant TAP1 expression was detected in 10/23 cervical carcinoma lesions and in 5/10 adjacent cervical intraepithelial neoplasia (CIN) lesions. All the lesions with low TAP expression also had reduced HLA class I expression. LOH was found in 7 out of 10 lesions with TAP loss. Mutation analysis detected no aberrations, but identified a polymorphism in the 5'-untranslated region (UTR) of the TAP1 gene in two lesions. This study shows that defective TAP expression in cervical carcinoma is often associated with LOH in the TAP region but not with mutations in the TAP1 gene.

Introduction

Immunological surveillance of tumour cells and virally infected cells is performed by cytotoxic T-lymphocytes (CTLs), which recognise aberrant peptides presented by human leukocyte antigen (HLA) class I molecules. HLA class I downregulation is caused by different molecular mechanisms and results in escape from CTL attack[1]. Antigen presentation by the HLA molecules to circulating CTLs requires the transporter associated with antigen processing (TAP). Endogenous proteins (viral or tumour associated products, or waste products from the cell itself) are degraded in the cytosol into smaller peptides. These peptides are subsequently transported by TAP. The latter consists of the subunits TAP1 and TAP2 that form a channel in the endoplasmatic reticulum (ER)-membrane[2]. In the ER, the assembly of the HLA class I heavy chain, the β_2-microglobulin (β_2m) light chain[3] and the peptides[4] is chaperoned by several proteins[5-8]. The newly formed complex is then transported via the Golgi network to the cell surface.

HLA class I downregulation is frequently associated with impaired TAP expression in various tumour types[9-12]. Limited knowledge is at hand concerning the molecular mechanisms underlying TAP loss. Deletion in the TAP1 gene has been described that leads to rapid degradation of its mRNA in a melanoma cell line[13,14] and recently mutations in the TAP1 and TAP2 genes have been found in colorectal carcinomas[15]. It has been suggested that in renal cell carcinoma TAP defects are caused by regulatory abnormalities[16]. Using real-time PCR it has been shown that in bladder cancer there is a coordinated transcriptional downregulation of the HLA class I antigen processing machinery, including TAP, which causes loss of HLA class I expression[17].

HLA phenotype alterations occur frequently in cervical cancer and its precursor lesions[18-23]. Koopman *et al.* described that this is caused by extensive loss of heterozygosity (LOH) at chromosome 6p21.3, partly in combination with mutations in the β_2m or HLA class I genes[22]. The TAP expression data from previous studies are inconsistent, with loss of TAP1 being reported in 0–50% of cases[24-27]. Moreover, limited information exists about the genetic mechanisms leading to loss of expression. A recent study of cervical carcinoma lesions has reported possible mutations in the TAP genes, but no sequence analysis was done and the loss of TAP expression was not studied[28].

These limited and contradictory results prompted us to investigate the loss of TAP in relation to HLA class I expression in invasive cervical carcinoma and adjacent cervical intraepithelial neoplasia (CIN) lesions. Furthermore, we examined possible causative mechanisms of altered TAP expression by performing LOH and gene mutation analysis on flow-sorted pure tumour and normal cell fractions from paraffin embedded cervical cancer tissue.

Material and Methods

Tissue Samples

Since 1989, Surinamese women with cervical carcinoma have been coming to the Leiden University Medical Centre (LUMC) at Leiden, the Netherlands, to have a radical hysterectomy (Wertheim operation). All the resected tumour tissue is stored in the tissue archive of the Pathology Department of the LUMC. All patients had a FIGO stage IB or IIa, which qualified them for the radical hysterectomy. Surinamese cervical cancer samples were selected for this study, because of the frequent loss at chromosome 6p that was previously observed[29]. In addition, cervical carcinomas of Surinamese women are usually of substantial size, which provided us with sufficient residual material for the extensive TAP1 gene sequence analysis.

From the group of Surinamese cervical cancer patients that were treated between 1989 and 2004, we selected the cases with invasive cervical carcinoma and an adjacent high-grade cervical intraepithelial neoplasia (CIN III) by reviewing the haematoxylin-eosin-stained slides. Precursor lesions connected to the cervical tumours were required to investigate the timing in addition to occurrence of TAP aberrations. We found 23 cases of cervical cancer, which were operated on between 1989 and 2004 that had an adjacent CIN lesion with sufficient tissue to investigate. From these 23, tissue blocks containing formalin-fixed, paraffin-embedded normal tissue and tumour tissue with an adjacent CIN lesion were selected.

Immunohistochemistry

Immunohistochemistry was performed on freshly cut, 3-μm thick, formalin-fixed, paraffin-embedded sections according to standard procedures[30]. Slides were incubated overnight with mouse monoclonal antibodies (mAbs) that are suitable for staining paraffin sections, the TAP1-specific mAb NOB-1 (S. Ferrone). This mAb is secreted by a hybridoma derived from the fusion of murine myeloma cells P3-X63-Ag8.653 with splenocytes from a BALB/c mouse immunised with partial length TAP1 recombinant protein (aa 434-735) and a key-hole limpet hemocyanin (KLH)-conjugated TAP1 peptide (aa 717-735). The specificity of the mAb was assessed by its reactivity with the corresponding antigen when tested in Western blotting with a lysate of lymphoid cells which express TAP1 and by the lack of reactivity with a lysate of the T2 cell line, which does not express these molecules[5,11] and anti-TAP2 (clone TAP2.17, Becton Dickinson Biosciences Pharmingen, San Jose, CA, USA). Furthermore, the mouse mAbs HCA2 and HC10 (Dr J. Neefjes, NKI, Amsterdam, the Netherlands) and the primary rabbit polyclonal anti-β_2m (A 072; DAKO, Copenhagen, Denmark) were used. The mAb HCA2 recognises a determinant expressed on β_2m-free HLA-A (excluding HLA-A24), HLA-B7301 and HLA-G heavy chains[31,32]. The mAb HC10 recognises a determinant expressed on all β_2m-free HLA-B and HLA-C heavy chains,

as well as on β_2m-free HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32 and HLA-A33 heavy chains[31,33].

Staining was scored semi-quantitatively by the quality control system proposed by Ruiter *et al*[34]. The intensity and percentage of positive tumour cells were determined. The intensity of staining was scored as 0, 1, 2 or 3, for absent, weak, clear or strong expression, respectively. The percentage of positive cells was scored as 0 for 0%; 1 for 1–5%; 2 for 5–25%; 3 for 25–50%; 4 for 50–75% and 5 for 75–100%. The sum of both scores was used to identify three categories of expression: normal expression (total score 7–8), partial loss (3–6), and total loss (0–2). In each tumour, stromal cells including lymphocytes served as a positive control for HLA class I and TAP expression.

Staining, Flow Cytometry and Sorting

Staining, flow cytometry and sorting of all formalin-fixed, paraffin embedded samples was performed as described previously with minor adjustments[35]. Multiple 0.6 mm punches from representative tumour areas of the paraffin blocks were treated with a combined mechanical/enzymatic method to obtain single cells. Cells were subsequently stained with a mAb mixture directed against keratin and vimentin, containing clones MNF116 (anti-keratin; DAKOCytomation, Glostrup, Denmark), AE1/AE3 (anti-keratin; Chemicon International Inc, Temecula, CA, USA) and V9-2b (anti-vimentin; Department of Pathology, LUMC)[36] or 3B4 (anti-vimentin; DAKOCytomation). Propidium iodide (PI) was used as a DNA stain.

Tumour and normal cell subpopulations were flow-sorted based on keratin and vimentin expression, respectively, combined with a gate on DNA content using a FACSVantage flow sorter (BD Biosciences). Flow-sorted cells (yields ranging from $2x10^5$ to $1x10^6$ cells) were collected in 5.0 ml Falcon™ tubes and centrifuged at 1000g for 10 min before DNA extraction[37].

LOH Analysis

DNA was extracted from sorted tumour cell subpopulations (keratin positive, vimentin negative) for LOH analysis. The microsatellite markers D6S2444 (UniSTS: 239054); TAP1 (3'-GGACAATATTTTGCTCCTGAGG-5' (F); 3'- GCTTTGATCTCCCCCCTC-5' (R)) and M2426 (3'- TTGTGGTTTCAGCTACTCAGG-5' (F); 3'-GTTTCTTTTCTTTCATTTGGCCTCTACTG-5' (R)) located in the TAP region on chromosome 6p21.3 were used. DNA extracted from the keratin negative, vimentin positive cell fractions was used as a normal DNA reference.

Standard PCR amplifications were performed as described[38]. All reactions were performed at least *in duplo*. The thresholds for retention of heterozygosity (ROH), "grey area" (allelic imbalance) and LOH were applied as previously empirically determined[37]. These were 0.76 – 1.3 (ROH), 0.58 – 0.75 or 1.31 – 1.69 (allelic imbalance) and 0.57 ≥ or 1.7 ≤ (LOH).

Sequence Analysis of the TAP Gene

The 11 TAP1 exons, including the exon-intron boundaries were amplified using the primers shown in **TABLE 1**. All primer positions were derived from the genomic sequence of the gene (Ensembl accession number: ENSG00000168394).

The only available DNA isolates were obtained from flow-sorted, paraffin-embedded material; we thus used several different primer sets for some exons in order to acquire easily amplifiable products. Sequencing was performed by the LGTC (Leiden Genome Technology Centre, the Netherlands). The obtained sequences were compared with DNA sequences of sorted autologous normal material (vimentin positive, keratin negative) and with the corresponding allele sequences from the Genome Database (http:/www.gdb.org).

TABLE 1

Primers used for sequence analysis of flow-cytometry sorted, paraffin-embedded tumour samples for mutations in the 11 exons of the TAP1 gene

		Forward Primer	Reverse Primer
Exon	1-1	ctggtgcaagtggaaaggca	ctggcgagaagctcagcca
	1-2	gccgctttcgatttcgcttt	gggcctgaagctccgggta
	1-3	ccgccagtaggggaggact	ggcgagaagtagcagtactgtcc
	1-4	caatggctagctctaggtgt	cacggcccagcggctca
	1-5	cccggagcttctctcgcat	cagctaatggcttcaaagcag
	1-6	cgctgggccgtgctctg	cagtgcagtagcctggtgctatc
	1-7	gttccgagagctgatctcatgg	agcctagaagccgacgcaca
	1-8	ggtcagggcggctctggaa	ttgccctgcgttccccttac
Exon	2	tctgactggaactgacctacttag	aacttccaactccctcatttg
Exon	3	aacacaccctgatcccctt	gaacagtacatggcgtataatg
Exon	4-1	gaacctgtctgattcacctcac	ccagagcatgatccccaa
	4-2	gtacctggtgcgaggcctat	gggagatgagggtctgtgtag
Exon	5-1	tgccaacccgtgtgacatct	ttccctaaacttctgggcttcg
	5-2	gaagctttgccaacgagga	gggaatgggtattcatcttca
Exon	6	ttgtggtctctttatagatttcag	cactggggagtgaaggtg
Exon	7-1	cctcactttcactattcttacct	aagggagtcaacagaccact
	7-2	tttgagtacctggaccgcac	gccagtggaatacagggagtg
Exon	8	gtgtgcttctctggcctcta	caagccacctgcttccata
Exon	9	ccttgttctatgactcttcatcat	ggctgggtggtgagatga
Exon	10	ggctataccgttctcatcttgg	ccattaagaagatgactgcctca
Exon	11-1	cggctctgacggtccgatg	tgcaccatggcccagtagca
	11-2	tctggaaggaggcgctatc	gaggagcttggaaaggaggt
	11-3	agctgcctccaggatgagtt	gctgatcatctttccgtaca
	11-4	ggtgtggccagcactctgaa	gtttggtgtgccggaaacat

Results

Loss of HLA Class I and TAP Expression in Cervical Carcinoma and CIN

Tissue sections with both tumour and adjacent CIN tissue were stained for HLA-A (HCA2), HLA-B/C (HC10), β_2m, TAP1, and TAP2 expression. The data are displayed in **FIGURE 1**, grouped based on the TAP1 expression of the samples: absent, weak and positive. Complex combinations of expression patterns for the different molecules studied were observed in the majority of cases. Only one case (S87) showed complete loss of TAP1 expression and resulting HLA-A and HLA-B/C loss of expression. In this case, β_2m was expressed and TAP2 was weakly expressed underlining the importance of TAP1 expression for stability of the HLA/β_2m/antigenic peptide complex and eventual HLA surface expression. In 9 additional tumour samples, TAP1 expression was weak. In one of these cases (S85) the adjacent CIN lesion was negative, in 3 cases (S84, S31, S04) the CIN followed the expression pattern of the tumour sample, while in 4 other cases (S86, S61, S08, S10) the CIN lesions were positive. In S71, the adjacent CIN showed weak TAP1 expression while the tumour was positive. In 5 of the 9 cervical carcinoma lesions with TAP1 downregulation, TAP2 was also weakly expressed (S85, S84, S31, S04, S86). Total

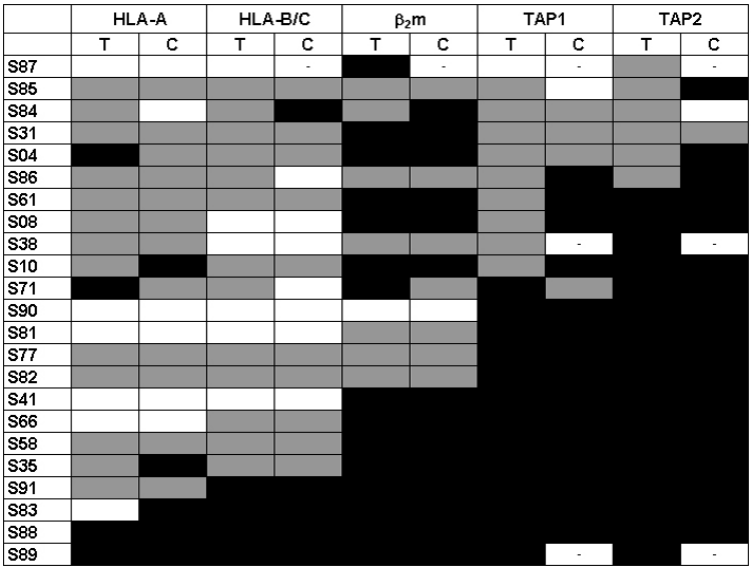

FIGURE 1

Expression patterns of HLA-A (HCA2), HLA-B/C (HC10), β2m, TAP1 and TAP2, grouped with regard to TAP expression. (T) Tumour; (C) CIN lesion. White squares represent loss of expression; grey squares- low expression; black squares- normal expression. (-) No data.

HLA class I expression was downregulated in 8 of the 9 cases with TAP1 defects (only case S04 showed positive expression for HLA-A). In 4 of those 9 cases β_2m expression was altered as well (S85, S84, S86, S38), which points towards cumulative negative effects of alterations in TAP and β_2m on HLA expression. In 12 (of the 23) cases, TAP expression was not affected. In only 1 case (S90), total HLA class I loss could be explained by total β_2m absence. Two tumours (S77, S82) showed weak total HLA expression in concordance with the weak β_2m expression observed, while in sample S81 weak β_2m expression was probably accompanied by a second hit at the HLA class I region on chromosome 6p21, as this case was negative for HLA expression. In cases S35 and S91, low expression of both HLA-A and HLA-B/C, and solely HLA-A, respectively, was found, indicative of possible genetic aberrations in these genes such as mutations or LOH. Tumour S83 was solely negative for HCA2 implying a specific mutation in the HLA-A gene. Two samples (S88 and S89) were positive for expression of all molecules studied. Expression of all studied molecules was not always concordant between tumour and adjacent CIN. In the majority of cases, the tumour sample showed weaker or absent expression as compared to the tumour-associated CIN (i.e. S86, S61, S08, S10 for TAP1 expression). In **FIGURE 2**, a representative case is shown stained with the 5 antibodies used.

LOH on Flow-Sorted Tumour Cells

The 23 tumour samples were all flow-sorted to obtain pure tumour cell fractions (keratin positive), assuring the precision and reliability of the LOH results (**FIGURE 3A**). Stromal cells and infiltrating lymphocytes are responsible for masking true LOH. These cells (vimentin positive) were also sorted and used as a normal control in all experiments. We performed LOH analysis only on the tumour cell fractions and not on the adjacent CIN cells as we were not able to obtain sufficient amounts of flow-sorted CIN material. Three markers that cover the TAP region on chromosome 6p21.3 were applied. A representative example of LOH for the TAP1 marker is shown in **FIGURE 3B**. In 4 cases none of the markers was informative and other 4 showed retention of heterozygosity for all markers (**FIGURE 3C**). LOH for at least 1 marker was found in 9 of the cases. The TAP1 marker, which is located in the TAP1 gene, was lost in 6 cases (S87, S04, S86, S61, S41, S91). The TAP2 gene is located in between the TAP1 marker and the D6S2444 marker but the latter is situated at approximately 100 kb from the TAP2 gene. This marker showed LOH in 5 cases (S04, S86, S61, S08, S10) but was also often non-informative (13 cases). When linking TAP protein expression to LOH, 2 cases with LOH had retained TAP expression. However, in 7 cases with LOH with at least 1 marker in the TAP region, TAP loss or weak expression was observed (S87, S04, S86, S61, S08, S38, S10) (**FIGURE 3C**).

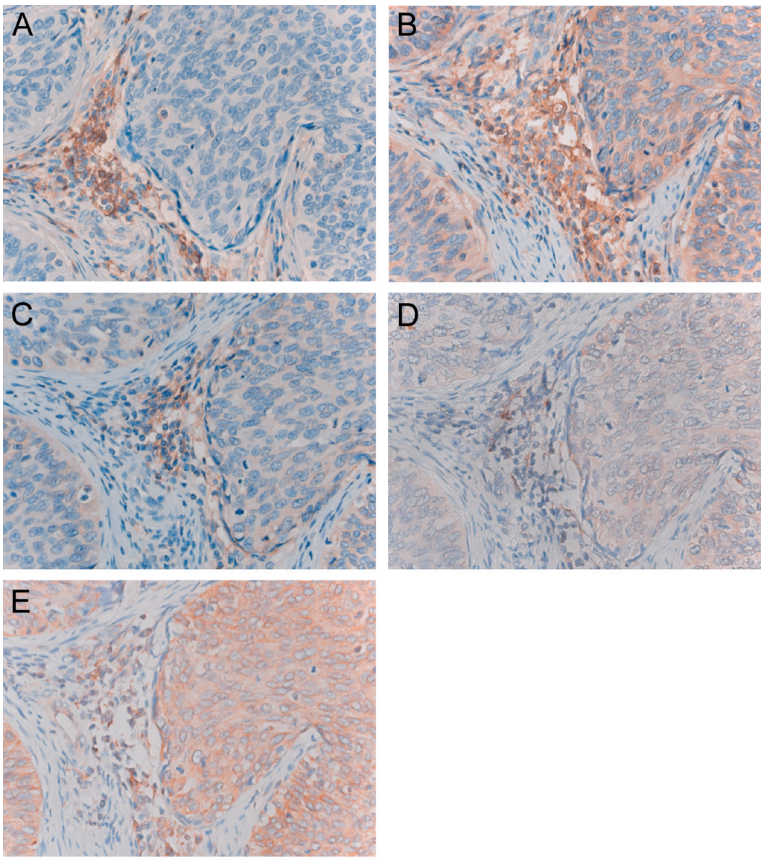

FIGURE 2

Immunohistochemical staining of a cervical carcinoma lesion (sample S87). Detail (400x magnification) of the same group of tumour cells, stained with TAP1 (negative) (**A**); TAP2 (positive) (**B**); HLA-A (**C**) and HLA-B/C (**D**) (weak cytoplasm, negative membrane); β2M (positive cytoplasm) (**E**).

Mutation Analysis

The 7 cases with loss of TAP expression and LOH with at least 1 marker located in the TAP region were selected for mutation analysis of the complete TAP1 gene (11 exons). To assure that no mutations could be missed as a result of contaminating normal cells present in the samples, sequencing was performed on the flow-sorted pure tumour cell fractions. Two cervical carcinomas carried a polymorphism located in the 5'-untranslate region (UTR) preceding exon 1 (Ensemble SNP annotation: rs3216794). A 1-nucleotide "C" deletion was observed in both the normal and tumour sorted cell fractions (data not shown). No mutations or other polymorphisms were found.

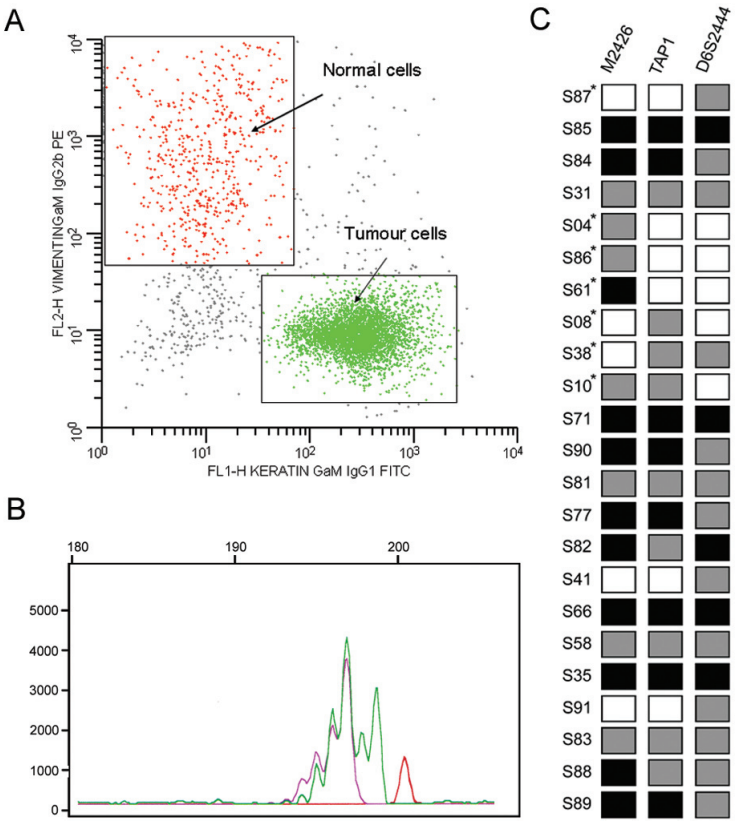

FIGURE 3

LOH analysis results. (**A**) A representative example of flow-sorting data of a cervical cancer sample (S41). The keratin positive (tumour, FITC-labelled) cells and the vimentin positive (normal, PE-labelled)) cells were flow-sorted and used in further analyses. (**B**) LOH results (S87) at marker TAP1 for tumour (pink, one peak) and normal (green, two peaks) sorted cell fractions (S87). A size marker is depicted in red. (**C**) The complete LOH data of the three microsatellite markers used per tumour sample, represented as ROH (black squares); LOH (white squares) and not informative (grey squares). The same order of samples is used as in **FIGURE 1**.

Discussion

In the present study, we determined the association between loss of TAP expression and loss of HLA class I in cervical carcinogenesis and examined possible causative molecular mechanisms to add to the hitherto limited knowledge of TAP aberrations in cervical cancer.

Even though loss of TAP expression has previously been reported and found to be associated with HLA class I downregulation in cervical carcinoma lesions, the reported

results are contradictory with 0-50% of the cases being negative[24-27]. Discrepancies are probably due to the frequently divergent scoring methods. In the majority of studies, cases containing less than 25% positive tumour cells are scored as negative[11,12,15,16]. Here, we applied the semi-quantitative scoring system as proposed by Ruiter *et al*.[34], which in our opinion gives a more accurate estimation of expression. Using this method downregulation of TAP1 expression was observed in 10/23 cases. In 6 of these 10 cases TAP2 expression was also low. None of the samples had loss of TAP expression throughout the whole tumour area with scattered nests of positive tumour cells. Although the group of patients described here is quite small, we were interested in determining TAP expression in cervical tumours with adjacent CIN lesions and these are scarce. Only some of the adjacent CIN lesions displayed low TAP expression, indicating that the moment of occurrence of these aberrations varies between cervical tumours. In several cases the altered TAP expression was more extensive in CIN than in the invasive tumour tissue. This could imply that the CIN and the tumour have a different clonal origin. In addition, probably only TAP negative CIN lesions could survive T-cell attack activated by the tumour's presence.

In cervical cancer loss of HLA class I cell surface expression is predominantly caused by extensive LOH at chromosome 6p21.3, where the HLA class I genes encoding the heavy chains of the HLA molecules are located[22,23]. Additional mutations in these genes have also been described[22]. The genes encoding the TAP1 and TAP2 molecules are located in the same region of chromosome 6p21[39]. Several studies have investigated the presence of mutations as well as regulatory and transcriptional abnormalities affecting the TAP genes in various tumour types[13-17,40]. A recent study on alterations of the TAP genes in cervical carcinoma was performed using single strand polymorphism PCR on blood and tumour samples[28]. The authors proposed that the major cause of TAP loss is the presence of TAP gene mutations. However, neither direct sequencing analysis nor expression analysis was performed to determine whether the loss of specific TAP alleles in fact leads to TAP protein loss.

To unravel the molecular mechanisms leading to loss of TAP expression in cervical cancer, we performed LOH analysis on flow-sorted tumour keratinocytes (keratin-positive) isolated from 23 paraffin-embedded cervical carcinoma samples using concurrently sorted, vimentin-positive normal cells as an autologous control. In cervical cancer, some of the tumour cells might express both keratin and vimentin, which would lead to a biased selection for the solely keratin-positive population using the flow-sorting method. Although such double positive cells were shown to have a higher proliferation rates and invasive potential in breast cancer[41], this does not seem to be the case in cervical tumours (manuscript in preparation). In addition, in the present group of patients the keratin-positive/vimentin-negative tumour cells represented the major subpopulation in the samples.

Seven of the tumours with downregulation of TAP expression were shown to have LOH using markers specific for the TAP genes. It is plausible to suggest that when one allele of

either one or both of the TAP genes is absent as a result of LOH, this will lead to aberrant TAP protein expression.

In addition, thorough sequence analysis of the TAP1 gene was also performed on flow-sorted pure tumour cell fractions from the 7 cervical carcinoma samples with altered TAP expression and LOH. Although the studied cases had heterogeneous low TAP expression, which might obscure the detection of mutations, previously colon tumours with similar TAP staining patterns were shown to carry mutations[15]. However in the present study no mutations were detected in any of the cases.

In summary, we detected altered TAP expression in a substantial number of cervical carcinoma to be associated with LOH of 6p21.3 where the TAP genes are located and not with mutations in TAP1. The applied flow-sorting procedure allowed us to perform precise molecular analysis of the tumours, without admixture of stromal cells, infiltrating lymphocytes and other normal cells, which are known to affect LOH analysis and to obscure mutation detection[42,43]. Currently, we are studying the presence of TAP polymorphisms in cervical carcinoma to determine whether LOH is associated with the retention of specific TAP alleles.

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Chapter 6

Physical Status of Multiple Human Papillomavirus Genotypes in Flow-Sorted Cervical Cancer Cells

Abstract

Multiple HPV infections have been detected in cervical cancer. To investigate the significance of multiple HPV infections we studied their prevalence in cervical cancer samples from a low-risk (Dutch) and a high-risk (Surinamese) population and the correlation of HPV infection with tumour cell aneuploidy. SPF_{10} LiPA was used for HPV detection in 96 Dutch and 95 Surinamese formalin-fixed cervical carcinoma samples. Samples with combined HPV 16/18 infections were sorted by flow cytometry and fluorescence in situ hybridisation was performed on the diploid and aneuploid subpopulations to detect HPV 16 and 18 genotypes simultaneously. Multiple HPV infections were present in 11/80 (13.8%) Dutch and 17/77 (22.1%) Surinamese carcinomas. Three cases had an HPV 16 and HPV 18 co-infection: in two cases, integrated HPV copies of HPV 16 or 18 were detected in the aneuploid fraction and in one case both HPV 16 and 18 were present solely as episomes. Based on our findings multiple HPV infections are present in cervical cancer samples from both high- and low-risk populations. Furthermore, multiple HPV types can be present in an episomal state in both diploid and aneuploid tumour cells, but integrated HPV genomes are detectable only in the aneuploid tumour cell subpopulations.

Introduction

Human papillomavirus (HPV) infection is a prerequisite for the development of cervical cancer (reviewed in[1]). Although HPV infections are common, the majority are transient and are cleared by the immune system[2]. When high-risk (HR-)HPV is persistently present, low grade cervical intraepithelial lesions (CIN) eventually progress to invasive cervical carcinoma[3]. HPV infects the basal epithelial cells and is frequently found in an episomal state in low- and high-grade CIN. It is generally thought that viral integration into the human genome occurs during cervical carcinogenesis[2,4]. Upon integration, the viral E2 repressor is disrupted, leading to continued expression of the E6 and E7 oncoproteins. They inactivate the p53 and pRb tumour suppressor proteins, leading to uncontrolled cell proliferation and ultimately to cancer[5]. Viral integration frequency was shown to increase with disease severity[4,6,7]. The two most common HR-HPV types found in cervical carcinoma are HPV 16 and HPV 18. While both HPV 16 episomes and HPV 16 integrated copies are able to transform normal keratinocytes, HPV 18 has been reported to be present mainly in the integrated form[6,8].

Fluorescence in situ hybridisation (FISH) has recently been used to investigate the physical state of HPV (episomal or integrated) in cells from cervical (pre)invasive lesions[9,10]. Several studies suggest that a diffuse nuclear signal is indicative of the presence of episomal HPV, while a punctate signal in the nucleus is characteristic of integrated HPV[11-13]. It was shown that the diffuse signal can be excluded by harsh pre-treatment protocols, allowing the clear detection of integrated HPV copies[10].

In addition to the physical state of HPV, abnormal cellular DNA content or numerical chromosome aberrations were suggested to be associated with the progression of CIN to cervical carcinoma[14,15]. Despite studies into whether HPV integration or DNA instability and aneuploidisation is the first step in malignant transformation, the sequence of events is still under debate[16,17]. Data on malignant transformation and progression as a result of multiple HPV infections are even more limited. Such multiple HPV infections are frequent in premalignant stages and have recently been detected in invasive cervical cancer[18-20].

In this study, we investigated the prevalence of multiple HPV infections in cervical cancer for a low-risk (Dutch) and a high-risk (Surinamese) population. The cases carrying a double HPV 16 and HPV 18 infection were analysed by performing FISH on flow-sorted pure tumour cell subpopulations to determine the integration status of the multiple HPV types in relation to tumour cell aneuploidy.

Material and Methods

Patient Samples

A total of 189 patients with invasive cervical carcinoma, FIGO stage IB or IIA, were included in this study. Patients were living in the Netherlands (n = 98), a low incidence area for cervical cancer, or Suriname (n = 99), a high incidence area for cervical cancer. Patients were diagnosed with cervical carcinoma between 1989 and 1995. All Dutch patients were treated in the Leiden University Medical Centre (LUMC), Leiden, the Netherlands and the tumour tissue was stored in the Pathology Department's archive. Of the Surinamese patients, 45 were treated in the LUMC and their tumour tissue was also kept in the LUMC laboratory. The other 49 patients were treated in Suriname and tissue samples were stored in the laboratory of the Pathology Department, Academic Hospital, Paramaribo, Suriname.

HPV Detection and Genotyping by SPF_{10} LiPA

DNA was isolated from formalin-fixed, paraffin-embedded biopsy samples as previously described[21]. Care was taken to prevent cross-contamination during preparation of the sections from the paraffin blocks. Beta-globin PCR was performed using primers RS40 and RS42[21] to determine whether the isolated DNA was suitable for amplification. The DNA was subjected to a short PCR fragment assay using the SPF_{10} primer set that amplifies a 65 base pair fragment in the L1 according to the manufacturer's instructions (Innogenetics, Gent, Belgium). Each experiment was performed with separate positive and several negative controls.

The presence of HPV was established using a microtitre plate-based hybridisation assay, and SPF_{10}-PCR products from HPV-DNA positive cases were directly genotyped by a reverse hybridisation line probe assay (LiPA (Innogenetics)). In this assay, 25 individual HPV genotypes (HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74) can be identified simultaneously.

On the HPV 16- and 18-positive specimens HPV-type 16 and 18 specific PCRs were performed as described previously[22].

Flow Cytometry and Sorting

Flow cytometry sorting of formalin-fixed, paraffin-embedded samples positive for multiple HPV types was performed as described previously[23]. Briefly, paraffin-embedded 60 μm sections were treated with a combined mechanical/enzymatic method to obtain single cells. Cells were then stained with a mix of monoclonal antibodies directed against keratin- and vimentin-containing clones MNF116 (anti-keratin; DAKO, Glostrup, Denmark), AE1/AE3 (anti-keratin; Chemicon International Inc., Temecula, CA, USA), and V9-2b (anti-vimentin; Department of Pathology, LUMC). A standard FACSCalibur (BD Biosciences,

San Jose, CA, USA) was used for flow cytometric analysis. Subsequently, flow sorting was performed using a FACSVantage flow-sorter (BD Biosciences). Tumour cells were sorted based on keratin and vimentin expression, combined with a gate on DNA content. Diploid and aneuploid tumour fractions and normal cell fractions were collected for DNA isolation; in addition, cell fractions were directly sorted separately onto glass slides for FISH processing.

Interphase FISH Analysis

FISH was modified to detect multiple HPV genotypes simultaneously. Interphase FISH analysis was performed on flow cytometry-sorted cell fractions using an adapted protocol for FISH on formalin-fixed, paraffin-embedded tissue[24]. Approximately 400 cells were sorted by flow cytometry directly onto glass slides that had been cleaned by rinsing in 96% ethanol. After spotting, the slides were dried overnight at room temperature to ensure cell adhesion. If needed, the slides were incubated in a 0.1M solution of $Na_2B_4O_7$ to permit swelling of the nuclei. Afterwards, the slides were rinsed in phosphate buffered saline and sterile water. The HPV 16 probe was labelled with digoxigenin and the HPV 18 probe was labelled with biotin (both purchased from PanPath, Science Park Amsterdam, Amsterdam, the Netherlands). Hybridisation and immunodetection were performed as described previously for nuclei isolated from paraffin-embedded material[24].

In each experiment, negative control slides spotted with sorted vimentin-positive nuclei not infected with HPV were stained simultaneously. Positive control slides of paraffin-embedded and sorted CasKi (HPV 16-positive), SiHa (HPV 16-positive), and HeLa (HPV 18-positive) cells were also included in all experiments. Centromere 1 (pUC1.77) and centromere 6 (p308) probes were used to ensure sufficient quality of the flow-sorted paraffin samples.

Results

HPV Detection and Genotyping

Beta-globin PCR was used as a control to ensure the quality of DNA in all samples. DNA in 2/98 (2.0%) of the Dutch samples and 4/99 (4.0%) of the Surinamese samples was found to be unsuitable for PCR and therefore excluded. Using SP10-LiPa PCR, similar HPV-positivity was found in both the low- and high-risk populations (**TABLE 1**). Among the Dutch HPV-positive samples, 11 different HPV types were detected, all of which were HR-HPV types. The Surinamese HPV-positive samples contained 17 different HPV types, of which two were low-risk HPV types. These low-risk HPV types were found in combination with (at least) one HR-HPV type.

TABLE 1
HPV Prevalence in Dutch and Surinamese Cervical Carcinoma Samples

HPV	Surinamese n (%)	Dutch n (%)	Total n (%)
Positive	77 (81.1)	80 (83.3)	157 (82.2)
Single	60 (77.9)	69 (86.2)	129 (85.3)
Multiple	17 (22.1)	11 (13.8)	28 (14.7)

TABLE 2
HPV type combinations detected in Dutch and Surinamese Cervical Carcinoma Samples

HPV type	Surinamese n (%)	Dutch n (%)	Total n (%)
16+18	1 (5.9)*	1 (9.1)*	2 (7.1)
16+33	1 (5.9)	-	1 (3.6)
16+52	2 (11.8)	-	2 (7.1)
18+31	-	2 (18.2)	2 (7.1)
18+33	-	2 (18.2)	2 (7.1)
18+52	1 (5.9)	2 (18.2)	3 (10.7)
16+18+52	1 (5.9)*	-	1 (3.6)
16+33+52	1 (5.9)	-	1 (3.6)
18+33+52	2 (11.8)	-	2 (7.1)
Other	8 (47.1)	4 (36.4)	12 (42.9)
Total	17 (100)	11 (100)	28 (100)

*, cases used in further analyses

Multiple HPV infections were more prevalent in the Surinamese cervical carcinoma samples, but this difference was not significant (OR 1.61, 95% CI 0.71 – 3.65) (**TABLES 1** and **2**). All but 2 samples were infected with at least 2 HR-HPV types. Occurrence and combinations of multiple infections are presented in **TABLE 2**. In 2 cases HPV 16 and HPV 18 double infection was detected and in 1 additional case next to HPV 16 and HPV 18 a co-infection with HPV 52 was present.

Integrated HPV in Flow-Sorted Aneuploid Cancer Cells

The 3 cervical tumours that were positive for both HPV 16 and HPV 18 (**TABLE 2**) according to LiPA genotyping were flow-sorted based on keratin expression and DNA content. Diploid and aneuploid tumour cell fractions were typed separately by HPV-specific PCR. The results are shown in **TABLE 3**.

In case 1, both HPV 16 and 18 were detected by LiPA in DNA extracted from the unsorted sample. Only HPV 16 was found after sorting in the diploid and aneuploid cell fractions, indicating that HPV 18 was not involved in malignant transformation in this

TABLE 3

Combined results of the various molecular methods performed for detection of HPV 16 and HPV 18 in cases with multiple infections

Cases	LiPA	Type-Specific PCR		FISH	
	Total sample	Sorted Diploid tumour cells	Sorted Aneuploid tumour cells	Sorted Diploid tumour cells	Sorted Aneuploid tumour cells
1	HPV16 + HPV18 +	HPV16 +	HPV16 +	HPV -	HPV16 +
2	HPV16 + HPV18 +	HPV16 + HPV18 +	HPV18 +	HPV -	HPV18 +
3	HPV16 + HPV18 +	X	HPV16 + HPV18 +	X	HPV -

X: no diploid subpopulation present.

tumour. FISH was positive for HPV 16 exclusively in the aneuploid tumour cell fraction (**FIGURE 1A** and **1B**). The punctate signal pattern observed is indicative of integrated HPV 16 DNA (**FIGURE 1B**).

Case 2 also consisted of diploid and aneuploid tumour cell fractions. While in the diploid fraction both HPV 16 and HPV 18 were detected by HPV-specific PCR typing, the aneuploid fraction was exclusively HPV 18-positive. FISH showed integration of HPV 18 in the aneuploid fraction, and no integration of either HPV 16 or HPV 18 DNA in the diploid fraction (**FIGURE 1D** and **1E**).

Case 3 was found to contain only an aneuploid tumour cell population. Both HPV 16 and HPV 18 were detected by HPV-specific PCR in the sorted tumour cells. However, FISH did not show integration of either of the two HPV types (**FIGURE 1G**).

Simultaneous FISH control experiments were performed using centromere 1 and centromere 6 probes to ensure sufficient quality of the sorted nuclei and were always positive for both diploid and aneuploid sorted tumour cell fractions (**FIGURE 1C, 1F** and **1H**). In addition, positive control cervical carcinoma cell lines containing integrated HPV 16 and HPV 18 copies were always positive (**FIGURE 2**).

Discussion

HPV is the causative agent of cervical carcinoma and approximately 80% of cervical cancer deaths occur in developing countries. The incidence is highest in Latin America, the Caribbean, Sub-Saharan Africa, and South Asia and considerably lower in North America and Western Europe[1]. It is known that the prevalence of HPV infection is tightly linked to cervical cancer incidence[1], and we have previously shown that Suriname is a high-risk

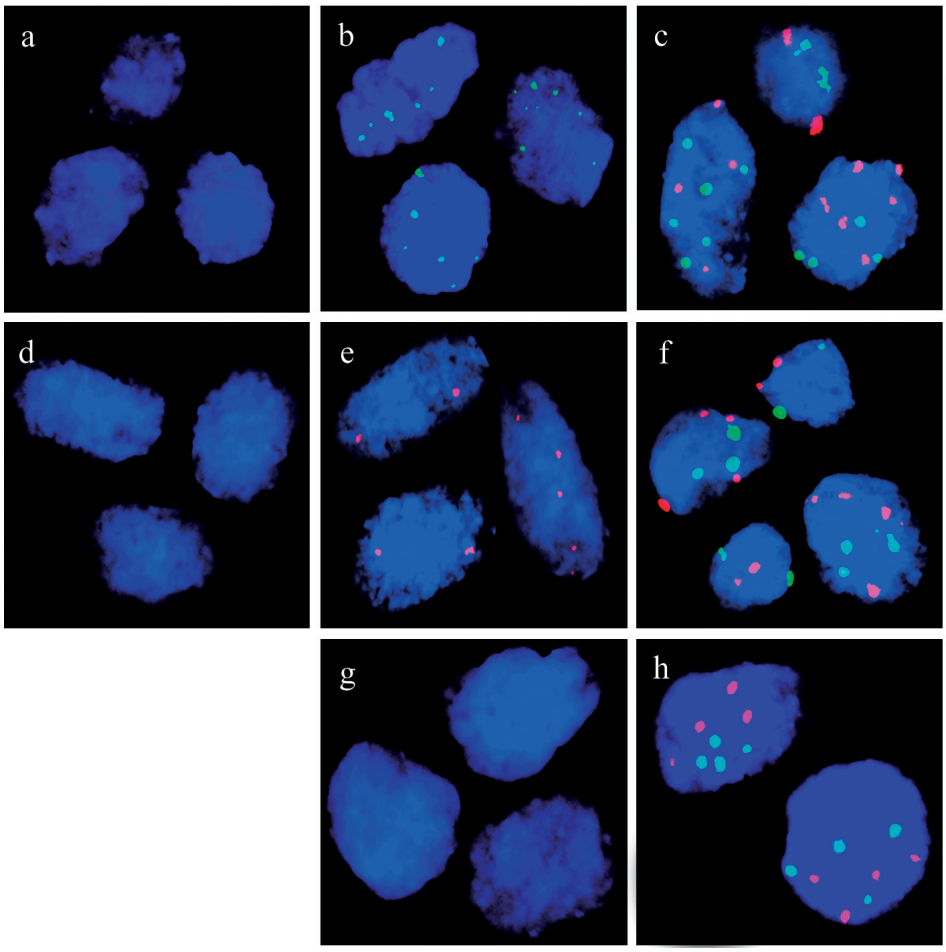

FIGURE 1

Interphase FISH on flow-sorted cervical carcinoma cells of the HPV 16/18 positive cases. Case 1 (**A**): the diploid cells are negative for HPV; (**B**) The aneuploid tumour cells show punctate signals for HPV 16 (green); (**C**) Control centromere 1 (red) and centromere 6 (green) signals. Case 2 (**D**): the diploid tumour cell fraction is negative for HPV; (**E**) The aneuploid tumour cells show punctate signals for HPV 18 (red); (**F**) Control centromere 1 (red) and centromere 6 (green) signals. Case 3 (**G**): the aneuploid tumour cell fraction is negative for HPV; (**H**) Control centromere 1 (red) and centromere 6 (green) signals.

country for HPV infection[25]. The prevalence of multiple HPV infections in our study group was 13.8% in Dutch and 22.1% in Surinamese cervical cancer samples, which confirms recent studies showing rates between 9% and 32%, depending on ethnicity[18-20]. Multiple infections were often seen in high-risk population studies, but high- and low-risk populations have not been compared. In our study, no significant difference was observed between the low-risk (Dutch) and high-risk (Surinamese) cases, although the odds of

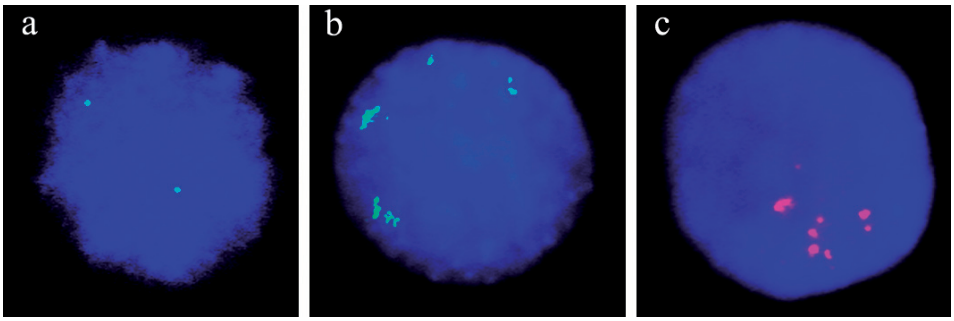

FIGURE 2

Interphase FISH on flow-sorted cervical cancer cell lines. (**A**) SiHa: 2 copies of HPV 16 are visible in green; (**B**) CaSki: multiple copies of HPV 16 are visible in green; (**C**) HeLa: multiple copies of HPV 18 are visible in red.

having multiple HPV infections was higher for the high-risk population (OR 1.61, 95% CI 0.71 – 3.65).

During HPV integration, HPV oncogenes E1 and/or E2 are frequently disrupted, while E6 and E7 are mostly preserved[4,5,26]. Some studies have used PCR techniques that compare expression of E2 and E6 to determine the integration prevalence. Other studies, however, have used other viral oncogenes for integration determination[27-29]. It remains difficult to accurately determine the incidence of HPV integration using PCR methods, due to the variability of disrupted and preserved viral oncogenes.

Here, we studied HPV integration using a FISH method that was modified to detect 2 different HPV types simultaneously. Previously, it was shown that harsh pre-treatment of samples washes away episomal forms of HPV DNA, leaving only the integrated HPV DNA to be detected by FISH[10]. This method appears very sensitive, but a detection threshold could account for missing single copy HPV infections. However the paraffin-embedded cell line SiHa (containing just 1-2 integrated copies of HPV 16[30]) was positive in all our experiments, showing that the technique can detect a single copy of integrated HPV DNA.

We investigated cervical carcinomas infected with both HPV 16 and HPV 18 more thoroughly. It was previously established that HPV 16 is present exclusively in episomal form in 30-70% of cervical cancers[6,8,31], while HPV 18 has been reported to be mainly integrated[6,32,33], indicating that these HPV types might have different biological characteristics. Badaracco *et al.* observed a remarkable increase from 20 to 54% in the prevalence of exclusively episomal forms of HPV 16 when coinfection with HPV 18 existed, but the physical status of HPV 18 was not investigated[8]. In the cases described in the current study, integration of either one of the HPV types was seen in 2 of 3 HPV 16- and HPV 18-positive tumours. In the third HPV 16- and HPV 18-infected tumour, no integration of either type was observed.

In addition to the variation in integration status of the HPV 16- and HPV 18-coinfected cervical carcinomas in our study, we also observed differences in DNA ploidy of the tumour cells. All three tumours had an aneuploid fraction, in all cases positive for HPV using HPV-specific PCR. The only case in which the aneuploid tumour cells tested positive for both HPV 16 and 18 using HPV-specific PCR was the case in which the HPV genome was not integrated, as indicated by the absence of a punctate FISH signal. In the other two cases, aneuploidy was associated with integrated HPV, either type 16 or 18.

Melsheimer *et al.* concluded that aneuploidisation precedes HPV integration in cervical carcinogenesis, as their study found that 19/20 lesions with integrated HPV were aneuploid[17]. Malignant progression of cervical neoplasia is associated with the expression of the HPV oncoproteins E6 and E7, and expression of these genes does not require HPV integration[34]. E6 and E7 deregulate cell cycle control mechanisms, create genomic instability, and can eventually cause aneuploidy[14,16]. However, near-diploid cervical carcinoma tissue with integrated HPV was observed previously. In two HPV integration studies using COBRA-FISH, evidence was found supporting both integration preceding aneuploidisation and vice versa[35,36]. COBRA-FISH is a method that is able to investigate tumour ploidy and HPV integration sites most elegantly and accurately. In one of these studies, Koopman *et al.* observed one aneuploid cell line with an episomal HPV pattern, but also a diploid cell line with HPV integration[35]. In the other study, Brink *et al.* studied fresh cervical carcinoma tissue and showed HPV integration in one diploid, two near-diploid, and one aneuploid cervical tumour sample[36]. These divergent results suggest that HPV integration can either precede or follow aneuploidisation.

In conclusion, cervical carcinomas that are infected with multiple HPV types are present in populations from both high- and low-risk areas for development of cervical cancer. The previously established association between HPV integration and aneuploidy is supported by our study; however, the succession of events seems to be flexible. Although a small number of samples were studied, this work shows that the use of archival, flow-sorted tumour subpopulations may contribute to our understanding of the variety of possible mechanisms leading to invasive cervical cancer.

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Chapter 7

General Discussion

Contents

Cervical carcinoma remains one of the leading causes of death from cancer among women worldwide[1]. Organised screening programmes aim to trace precursor lesions in order to reduce cervical cancer incidence. Human papillomavirus (HPV) is a necessary cause for cervical carcinogenesis. Most HPV infections are cleared and mild cervical abnormalities regress because of an efficient cellular immunity. A failing immunological surveillance eventually results in the development of cervical cancer.

This thesis describes and discusses the above mentioned aspects in cervical carcinogenesis. Several topics that are touched in the chapters are highlighted at this point in a general discussion.

1 Surinamese Environment and Cervical Cancer

Developing countries are high-risk areas for cervical cancer of which the incidence rates differ worldwide[1-5]. Suriname is such a high-risk area[6]. Both endogenous and exogenous factors can influence development of cervical (pre)malignancies and differences in incidence rates between geographical areas[7]. Important exogenous or environmental factors are HPV, screening history and sexual lifestyle[7-9]. HPV infection is more common in high-risk areas for cervical cancer[10-15], including Suriname[16]. A higher prevalence and viral load and different (more virulent) HPV type variants in a high-risk area might cause an increased risk of cervical cytological abnormalities, as suggested recently[17,18]. High HPV exposure could be associated with a sexual lifestyle encouraged by the Surinamese culture, as was established in several other populations[9,19-21]. Possible promiscuity of males or females clearly increases the risk of acquiring an HPV infection and it is suggested that the relatively young age at first intercourse could intensify the susceptibility to HPV persistence.

The high cervical carcinoma incidence in Suriname is reflected in the high prevalence of moderate and severe dysplasia which was observed in cervical smears from the first organised cervical screening programme in Suriname[Chapter 2]. The significance of environmental factors for differences in the geographical incidence of cervical cancer and its precursors was emphasised by a decreased prevalence of dysplasia in Surinamese immigrants in the Netherlands, a low-risk area for cervical cancer[Chapter 3]. This significance is also supported by studies comparing risk factors in areas with different cervical cancer incidences[18,22].

The majority of the decline in cervical carcinoma incidence rates in developed countries is attributed to the implementation of organised screening programmes[5,23,24]. The protective effect of previous screening operates independently from HPV[9,22]. An absent or an only recently started organised screening programme for cervical cancer could therefore explain a high prevalence of cervical lesions in an area, in this case Suriname.

In addition to the stated risk factors, several cofactors for progression of cervical HPV induced lesions have been reported. These include smoking, oral contraceptives, parity[7,8,20] and exposure to carcinogens in the household environment[9].

Along with the above mentioned environmental factors, endogenous aspects that mainly consist of immunological and genetic characteristics strongly influence the course of cervical carcinogenesis.

2 Significance of Multiple HPV Infections in Cervical Cancer

As said, premalignancies are mainly induced by HPV infection and persistence of oncogenic HPV is imperative for progression to cervical carcinoma. HPV persistence allows for expression of the HPV oncogenes E6 and E7, which is associated with the malignant progression of cervical neoplasia. The oncoproteins E6 and E7 deregulate cell cycle control mechanisms, create genomic instability, and can eventually cause aneuploidy[25,26].

In the multi-step cervical carcinogenesis with environmental, immunological and genetic factors, the role of multiple HPV infections is not immediately obvious. Until recently it was thought that multiple HPV infections were only present in premalignant cervical lesions, but now we know that invasive cervical tumours can also be infected by multiple HPV types. There appears to be no significant difference between high- and low-risk areas for cervical cancer, although the odds of having multiple HPV infections are higher in a high-risk population[Chapter 6].

The significance of multiple HPV infections in cervical carcinoma can be viewed in combination with viral integration and aneuploidy status. HPV integration is an important step in cervical carcinogenesis, but is not always necessary for the expression of E6 and E7[27]. It does, however, secure viral persistence, which is a prerequisite for expressing the viral oncogenes. Previously, HPV integration was investigated with PCR-based techniques, estimating integration by comparing the copy numbers of an often deleted (E1/E2) and a mostly preserved (E6/E7) viral oncogene. Recently several studies concerning HPV integration have been published that utilised fluorescence in situ hybridisation (FISH), which has the advantage of imaging episomal as well as integrated HPV. Most cervical carcinomas seem to have HPV present in the integrated form, occasionally accompanied by HPV episomes[28,29].

As stated above, the ultimate result of the deregulation of cell cycle mechanisms by the HPV oncoproteins is aneuploidy. It could be hypothesised that aneuploidisation occurs after viral persistence, making the tumour clone unstable and herewith facilitating HPV integration. This is suggested by Melsheimer *et al.* who showed that 19 out of 20 aneuploid lesions had integrated HPV[30]. However, one could also argue that viral integration increases genomic instability inducing aneuploidy. Evidence for both mechanisms was observed previously[31,32].

What role do multiple HPV infections have in cervical carcinogenesis? Apparently it is favourable for some cervical tumours to be infected with multiple HPV genotypes. An additional HPV type might result in sufficient oncogenicity even without viral integration in some tumours. In two out of three HPV 16/18 co-infected cervical carcinomas, we found viral integration of one type in the aneuploid fraction, but the third case showed presence of both types without HPV integration[Chapter 6].

To summarise, expression of the viral E6/E7 oncogenes does not require HPV integration [27], which is probably one of the main reasons that the timing of HPV integration in cervical carcinogenesis can vary. The presence of multiple, possibly synergistic, high risk-HPV genotypes could lead to extensive expression of the viral E6/E7 oncogenes. Aneuploidy weakens the genome and therefore enhances oncogenicity of a tumour in general as well as the integration of HPV. The main conclusion seems to be that there are multiple paths which can lead to progression of a cervical tumour, probably depending on individual immunogenetic and available environmental factors.

3 Immunogenetic Heterogeneity in Cervical Cancer

The cellular immune system is able to eliminate viruses and virus induced lesions. HPV induced cervical lesions are associated with a failing immunological surveillance which is performed by cytotoxic T-lymphocytes (CTLs), activated when human leukocyte antigen (HLA) class I antigen presents aberrant peptides. In addition, tumour progression appears to be facilitated by altered expression of cytokines among which are interferon and several interleukins, leading to decreased local cellular immunity[33-35].

Loss of HLA surface expression occurs frequently in cervical (pre)neoplasia[36-40]. The nature and frequency of HLA class I antigen loss mechanisms were elegantly studied in a group of freshly sorted cervical cancer samples and it was established that altered HLA class I expression was frequent, diverse, mainly caused by genetic changes and combined with widespread tumour heterogeneity[40]. The diversity and heterogeneity of HLA class I aberrations are illustrated by the expression patterns observed in cervical tumours and adjacent cervical intraepithelial neoplasia (CIN) lesions[Chapter 5]. Strong, weak and absent expression of HLA class I was observed in the samples, often varying within the cases. In most patients, CIN and invasive tumour samples provided similar results, supporting the hypothesis that both are from the same clonal process and demonstrating that HLA class I loss is an early event in cervical carcinogenesis. The failure to express HLA class I could result from loss of heterozygosity (LOH) at 6p21.3 in combination with a locus-restricted event in this area. Mutations in the HLA class I genes and in β_2m were described previously[40]. Larger deletions in the HLA class I area were not observed using FISH[Chapter 5]. It is likely that the HLA class I aberrations allow the premalignant CIN lesion to escape

immune surveillance and progress to invasive cancer.

Not all cases with loss of HLA class I expression can be explained by genetic defects in the HLA class I or β_2m genes. Low transporter associated with antigen processing (TAP) expression has previously been reported and was found to be associated with loss of HLA class I expression in cervical carcinomas[41-43], but until recently information about the underlying mechanisms was limited. Previously reported TAP mutations in the majority of cervical carcinoma samples emerged to be polymorphisms and LOH[44]. In our study altered TAP expression was observed in more than 40% of the cases, in two cases accompanied by a 1 bp deletion in the 5'-UTR[Chapter 6]. Only some of the adjacent precursor lesions displayed loss of TAP expression, indicating that the timing of TAP downregulation varies between cervical tumours. In several cases the loss of TAP expression was more extensive in CIN than in the invasive tumour tissue. This could implicate different clonal origins or the fact that only TAP negative CIN lesions survived T-cell attack activated by the tumour's presence. All samples with altered TAP expression displayed a heterogeneous staining pattern of scattered nests of TAP positive among TAP negative tumour cells, even in the cases with the 5'-untranslated region (UTR) somatic mutation and LOH. This type of pattern probably results from aberrations in regulation rather than from clonal expansion of TAP negative and positive tumour cell populations. Most TAP and associated HLA class I aberrations can be upregulated through interferon stimulation[45,46], although structural TAP alterations in tumour cells have been established recently[47-49]. TAP defects can diminish the HLA class I cell surface expression, but there is increasing evidence that an effective antiviral defence can occur via TAP independent mechanisms as well[50-54].

4 Conclusions

In the present thesis we focused on multiple aspects of cervical carcinogenesis in developed and developing countries. Several conclusions can be made based on the studies presented. The high cervical carcinoma incidence in Suriname is reflected in the high prevalence of moderate and severe dysplasia which was observed in a sample of cervical smears from the first organised cervical screening programme in Suriname. This can be attributed to the absence of an organised screening programme for cervical cancer until recently, which is associated with high prevalence of cervical lesions in an area. In addition, the decreased prevalence of dysplasia in first-generation Surinamese immigrants in the Netherlands illustrates the significance of environmental factors for differences in the geographical incidence of cervical cancer and its precursors.

Looking at endogenous aspects of cervical carcinogenesis it is important to realise that the timing of HPV integration in cervical carcinogenesis can vary, but the viral oncogenes always need to be expressed. The cellular immune system recognises HPV infected

lesions. Selective pressure results in HLA defective tumour cells and a failing immune surveillance increases cervical tumour progression. The frequent HLA class I aberrations occurring in cervical carcinoma are diverse and heterogeneous and partly associated with TAP alterations.

Hitherto, the largest decrease in cervical cancer incidence has been accomplished by organised cervical screening programmes. The success of organised screening programmes is conditional upon a high response rate and regular screening intervals, which unfortunately remains very difficult to realise in developing countries where cervical cancer is the leading cancer among women. In addition, the effect of successfully implemented screening programmes can first be observed after some time (decades). The large number of HPV vaccination studies illustrates the current focus in cervical cancer research. Prophylactic HPV vaccines show a protection of 70% against the high-grade cervical lesions. A prophylactic HPV vaccine seems a promising solution in the near future for developing countries in establishing a substantial decrease in cervical carcinoma incidence. However, the frequently aberrated immune system in women with cervical cancer suggests a difficulty in establishing an effective immunisation by therapeutic HPV vaccines, which should be taken into account in further research concerning these vaccines.

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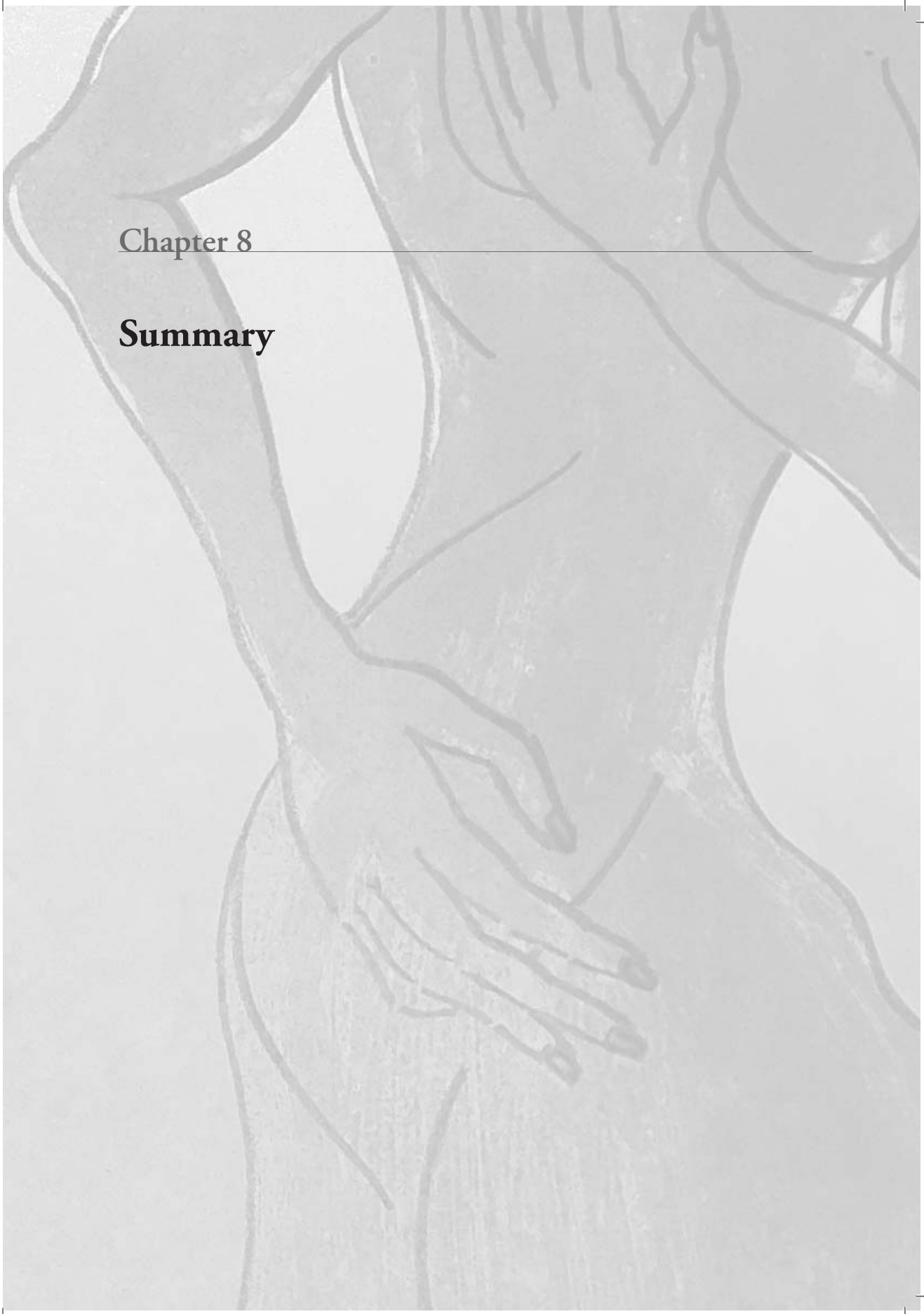


Chapter 8

Summary

In **CHAPTER 1** an introduction to several aspects of cervical cancer, HPV and their relation with immunology is given. It took almost one-and-a-half centuries from the first mention of a possible relationship between sexual intercourse and cervical cancer and the epidemiological evidence that the sexually transmittable HPV is the unifying risk factor of cervical cancer. The tumour process starts in the cervical transformation zone and develops through several stages to cervical carcinoma. Treatment of premalignant and early stages is often successful, but advanced stages are more difficult to cure. By introducing organised, population-based screening programmes developed countries have effectively reduced cervical carcinoma incidence and mortality rates. Developing countries remain areas where high incidences and prevalence of advanced stages are observed. Approximately 80% of the women worldwide are estimated to experience an HPV infection at least once, but the vast majority is able to clear the infection. HPV and HPV induced lesions are eliminated by the cellular immune system when successfully presented on HLA molecules. Genetically caused HLA aberrations can obviously disturb this process and this is thought to be of great significance in cervical carcinogenesis. This complicates the development of preventive and therapeutic HPV vaccines, the focus of much research. In the mean time a great deal of effort is put into implementing organised screening programmes in less economically developed (high-risk) countries.

The outline of this thesis is presented, which addresses epidemiological, immunogenetic and viral aspects in premalignant and invasive cervical lesions.

In **CHAPTER 2** we determined the prevalence of cytological abnormalities in cervical smears of women attending the first organised screening programme in Suriname and compared the prevalences in four Surinamese ethnicities with different cervical carcinoma incidence. Papanicolaou staining and cytological screening were performed on 807 cervical smears taken from Maroons, Amerindians, Javanese and Hindustani. Cervical cytological abnormalities were detected in 13.4% of the assessable smears, of which 2.6% were moderate and severe dysplasia. The cytological abnormalities varied between the ethnicities. In the smears of the Maroons significantly more cytological abnormalities were detected. We observed a high prevalence of moderate and severe dysplasia in all ethnicities, which correlates with the high cervical carcinoma incidence in Suriname. A significantly higher prevalence of mild abnormalities in the Maroons was seen, which did not reflect the relatively low cervical cancer incidence in this ethnicity. However, this can feasibly be explained by the possibility that these women have a different sexual lifestyle, leading to a higher prevalence of transient HPV infection.

Incidence rates of cervical cancer and its precursors vary considerably and are influenced by endogenous and exogenous factors. In **CHAPTER 3** we compared cytological abnormality incidence rates from a high-risk population in the original high-risk area with those

of women from this high-risk population who have immigrated to a low-risk area to give insight in the significance of these factors. Smears collected from Surinamese women attending the Surinamese screening programme and smears collected from immigrant Surinamese women attending the Dutch screening programme were cytologically analysed using the Dutch microscopical coding system KOPAC. The age-adjusted odds of having dysplasia were higher for Surinamese women living in Suriname versus Surinamese immigrant women and increased with increasing stage of atypical changes. We concluded that fewer cases with dysplasia are present in a high-risk population that has immigrated to a low-risk area for cervical cancer than in the high-risk population continuously living in a high-risk area. This finding emphasises the importance of environmental factors.

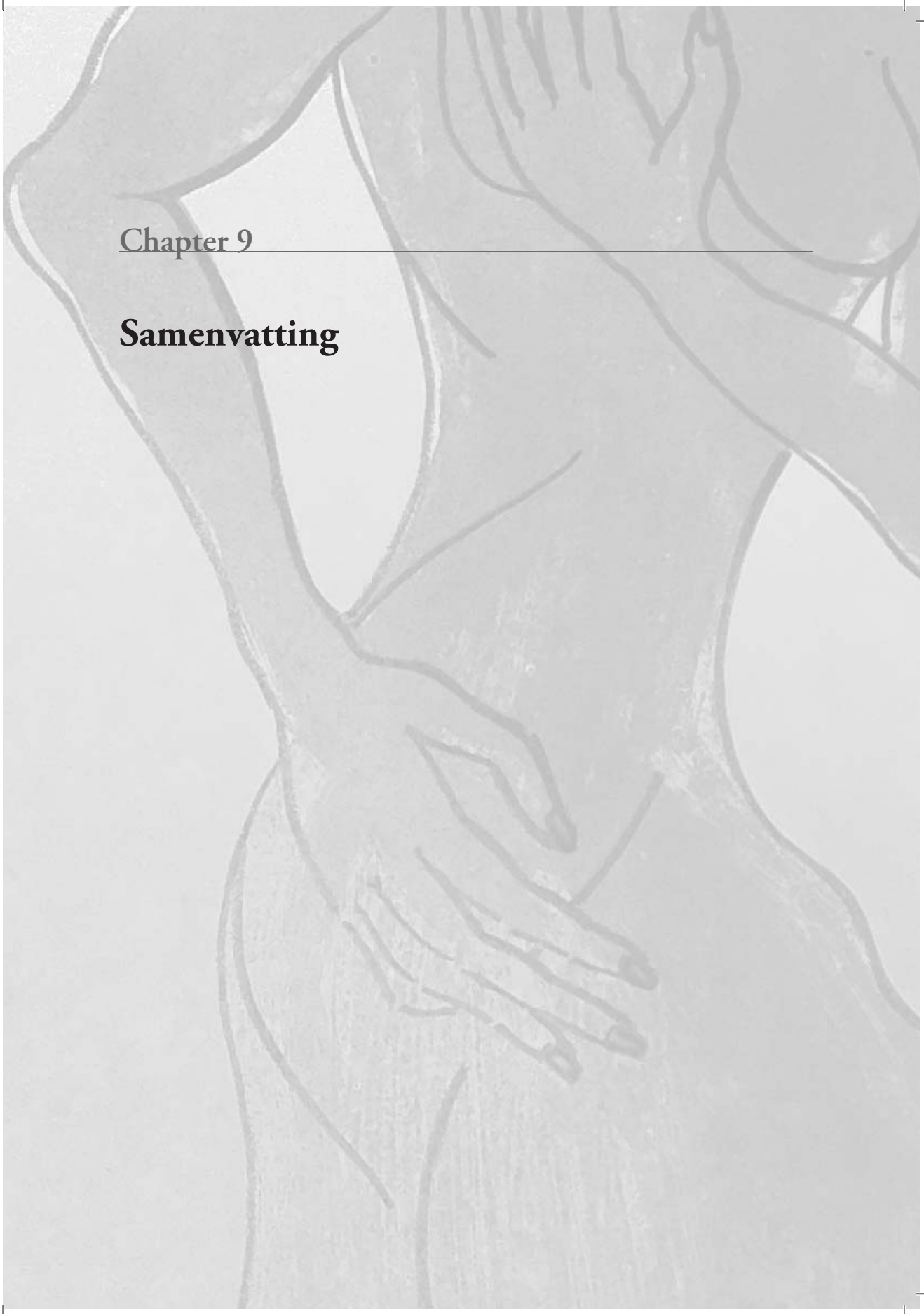
Loss at chromosome 6p21.3, the human leukocyte antigen (HLA) region, is the main cause of HLA downregulation, occurring in the majority of invasive cervical carcinomas. In **CHAPTER 4** we investigated timing, frequency and mechanism of HLA class I downregulation in cervical carcinogenesis. To identify the stage of tumour development at which HLA class I aberrations occur, we selected 12 patients with cervical carcinoma and adjacent cervical intraepithelial neoplasia (CIN). Including the precursor lesions in our study permitted us to add to the current knowledge of HLA aberrations in invasive cervical carcinoma. We investigated HLA class I and β_2-microglobulin expression by immunohistochemistry in tumour and adjacent CIN. Loss of heterozygosity (LOH) was studied using microsatellite markers covering the HLA region. Fluorescence in situ hybridisation (FISH) with HLA class I probes was performed to investigate the mechanism of HLA loss. Immunohistochemistry showed absent or weak HLA class I expression in 11/12 cases. In 10 of these 11 cases downregulation occurred in both tumour and CIN. In 9/12 cases LOH was present for at least one marker in both tumour and CIN, 1 case showed only LOH in the CIN lesion and 1 case showed retention of heterozygosity (ROH) for all markers in both tumour and CIN. We concluded that HLA class I aberrations occur early and frequently in cervical carcinogenesis. This might allow premalignant CIN lesions to escape immune surveillance and progress to invasive cancer.

Loss of expression of the transporter associated with antigen processing (TAP) can influence HLA membrane expression which is frequently down-regulated in cervical cancer and its precursors. HLA class I molecules activate T-cells by antigen presentation and are therefore important for immunological surveillance. To add to the hitherto limited knowledge of molecular mechanisms underlying TAP loss in cervical cancer we investigated TAP expression, LOH and possible TAP mutations in **CHAPTER 5**. To identify the timing of changes in TAP expression 23 cervical carcinomas and adjacent precursor lesions were stained with HLA-A-, HLA-B/C-, β_2-microglobulin-, TAP1- and TAP2-specific MoAbs. TAP1 was not detectable in 10 out of 23 cervical carcinomas and 5 out of 10 adjacent CIN

lesions. All the lesions with low TAP expression also had altered HLA class I expression. To be able to separate tumour and non-tumour cells, cervical carcinoma samples were sorted by flow-cytometry and were subsequently analysed for LOH with markers in the TAP region on chromosome 6p21.3. LOH was found in 6 of the 10 lesions with TAP loss. Mutation analysis was then performed on these cases. In two cases we detected a polymorphism in the 5'-untranslated region (UTR) of the TAP1 gene. No mutations were detected. This study shows that there is altered TAP expression in a substantial number of cervical carcinomas. The underlying mechanism seems to be LOH in the TAP region, which is not accompanied by a mutation. In all cases with low TAP expression HLA class I loss was concomitantly detected, which supports previous reports on a strong association between TAP aberrations and loss of HLA class I expression.

Human papillomavirus (HPV) is a prerequisite for the development of cervical cancer. It has been established that multiple HPV infections are common in premalignant stages. Recently, multiple HPV infection in invasive cervical cancer has also been determined. In **CHAPTER 6** we investigated the significance of multiple HPV infections by studying their prevalence in cervical cancer samples from a low-risk (Dutch) and a high-risk (Surinamese) population and the correlation of HPV infection with tumour cell aneuploidy. SPF_{10} LiPA was used for HPV detection and typing in 96 Dutch and 95 Surinamese cervical carcinomas. Subsequently, samples with combined HPV 16/18 infections were sorted by flow cytometry and both diploid and aneuploid tumour cell fractions were HPV-typed by HPV 16- and HPV 18-specific PCR. HPV integration was investigated on the sorted cervical carcinoma cells. Fluorescent in situ hybridisation (FISH) on paraffin embedded tissue was modified to detect HPV 16 and 18 genotypes simultaneously and was performed on the sorted samples. Multiple HPV infections were present in 13.8% Dutch and 22.1% Surinamese HPV positive cervical carcinoma lesions. Three cases carried an HPV 16 and HPV 18 co-infection: in two cases, integrated HPV copies of either HPV 16 or 18 were detected in the aneuploid fraction, and in the third case both HPV 16 and 18 were present solely as episomes. These results show that multiple HPV infections are present in cervical cancer samples from both high- and low-risk populations. Multiple HPV types can be present in an episomal state in both diploid and aneuploid tumour cells, but integrated HPV genomes were detected only in the aneuploid tumour cell subpopulations.

Conclusions that were drawn and hypotheses that were developed are put into perspective in **CHAPTER 7**.



Chapter 9

Samenvatting

In **CHAPTER 1** wordt een introductie gegeven in verschillende aspecten van cervix carcinoom, HPV en hun relatie met immunologie. De tijd tussen de vermelding van een mogelijk verband tussen seksueel verkeer en baarmoederhalskanker en het epidemiologisch bewijs dat het seksueel overdraagbare HPV de centrale risico factor voor cervix carcinoom is, beslaat bijna anderhalve eeuw. Het tumorproces begint in de cervicale transformatiezone en ontwikkelt zich via verschillende stadia tot cervix carcinoom. Behandeling van patiënten met premaligne en vroege stadia is vaak succesvol, maar patiënten met gevorderde stadia zijn moeilijker te genezen. Door het introduceren van georganiseerde bevolkingsonderzoeken hebben ontwikkelde landen de cervix carcinoom incidentie en mortaliteit effectief gereduceerd. Ontwikkelingslanden blijven gebieden met een hoge incidentie en prevalentie van cervix carcinoom patiënten met gevorderde stadia. Geschat wordt dat ongeveer 80% van alle vrouwen minimaal eenmaal een HPV infectie doormaakt, van wie de overgrote meerderheid in staat is het virus te klaren. HPV en HPV-geïnduceerde laesies worden door het cellulaire immuunsysteem geëlimineerd als ze tenminste correct worden gepresenteerd op HLA moleculen. HLA defecten van genetische oorsprong kunnen dit proces verstoren.

Vervolgens wordt de opzet van dit proefschrift gepresenteerd, dat epidemiologische, immunogenetische en virale aspecten van cervix carcinoom en zijn voorstadia bespreekt.

In **CHAPTER 2** beschreven we de prevalentie van cytologische afwijkingen in cervix uitstrijkjes, afkomstig van vrouwen die deel hebben genomen aan het eerste georganiseerde bevolkingsonderzoek in Suriname en vergeleken we de prevalenties van vier Surinaamse etniciteiten met een verschillende cervix carcinoom incidentie. Papanicolaou kleuring en cytologische screening worden uitgevoerd op 807 cervix uitstrijkjes afgenomen bij Marrons, Amerindianen, Javanen en Hindustanen. Cervicale cytologische afwijkingen werden vastgesteld in 13.4% van de beoordeelbare smears. Hiervan was 2.6% matige tot ernstige dysplasia. De prevalentie van cytologische afwijkingen varieerde tussen de etniciteiten. In de uitstrijkjes van de Marrons worden significant meer cytologische afwijkingen aangetoond. We stelden een hoge prevalentie van matige en ernstige dysplasie vast in alle etniciteiten, hetgeen correleert met de hoge cervix carcinoom incidentie in Suriname. Bij de Marrons werd een significant hogere prevalentie van milde afwijkingen gezien, hetgeen niet correspondeert met de relatief lage cervix carcinoom incidentie bij deze etniciteit. Dit zou wel kunnen passen bij een mogelijke andere seksuele levensstijl van deze vrouwen, hetgeen zou kunnen leiden tot een hogere prevalentie van voorbijgaande HPV infecties.

Incidentiecijfers van (voorstadia van) cervix carcinoom variëren aanzienlijk. Dit wordt beïnvloed door endogene en exogene factoren. In **CHAPTER 3** vergeleken we incidenties van cytologische afwijkingen van een hoog-risico populatie in het oorspronkelijke gebied met die van vrouwen van deze hoog-risico populatie die zijn geïmmigreerd naar

een laag-risico gebied om inzicht in het belang van deze factoren te krijgen. Uitstrijkjes afkomstig van Surinaamse vrouwen die deelnamen aan het Surinaamse bevolkingsonderzoek en uitstrijkjes afkomstig van vrouwelijke Surinaamse immigranten in Nederland werden cytologisch geanalyseerd, gebruikmakend van het Nederlandse microscopische coderingssysteem KOPAC. De leeftijdsspecifieke odds op het hebben van dysplasie waren hoger voor Surinaamse vrouwen die in Suriname wonen dan voor de naar Nederlands geïmmigreerde Surinaamse vrouwen en stegen met hogere stadia van atypie. Wij concludeerden dat er minder gevallen met dysplasie vóórkomen in een hoog-risico populatie die is geïmmigreerd naar een laag-risico gebied voor cervix carcinoom dan in een hoog-risico populatie die is blijven wonen in het hoog-risico gebied. Dit benadrukt het belang van omgevingsfactoren.

Verlies op chromosoom 6p21.3, het gebied van het humane leukocyten antigeen (HLA), is de hoofdoorzaak van HLA downregulatie, hetgeen bij het merendeel van de cervix carcinomen vóórkomt. In **CHAPTER 4** onderzochten we timing, frequentie en mechanisme van HLA klasse I downregulatie in de cervicale carcinogenese. Om het tumorstadium waarin de HLA klasse I aberraties optreden te identificeren, selecteerden we 12 patiënten met cervix carcinoom en aangrenzende cervicale intraepitheliale neoplasie (CIN). Het includeren van voorstadia in onze studie gaf ons de mogelijkheid om iets toe te voegen aan de actuele kennis van HLA aberraties in invasieve cervix carcinoom. Gebruik makend van immuunhistochemie onderzochten we HLA klasse I en β_2-microglobuline expressie in tumor en aangrenzende CIN. Verlies van heterozygositeit (LOH) werd onderzocht met microsatellite markers die het HLA gebied besloegen. Fluorescent in situ hybridisatie (FISH) met HLA klasse I probes werd uitgevoerd om het mechanisme achter het HLA verlies te ontrafelen. In 11 van de 12 gevallen liet immuunhistochemie afwezige of zwakke HLA klasse I expressie zien. In 10 van deze 11 gevallen kwam downregulatie in tumor en aangrenzende CIN voor. In 9 van de 12 gevallen werd LOH gezien met tenminste één marker in tumor en CIN, in één geval werd alleen LOH in de CIN laesie gedetecteerd en in één geval werd retentie van heterozygositeit voor alle markers in zowel tumor als CIN gezien. We concludeerden dat HLA klasse I aberraties vroeg en vaak in de cervicale carcinogenese optreden. Dit zou premaligne CIN laesies een mogelijkheid kunnen geven om aan immuunsurveillance te ontsnappen en te ontwikkelen tot cervix carcinoom.

Verlies van expressie van de "transporter associated with antigen processing" (TAP) kan de HLA membraan expressie beïnvloeden, die frequent is down gereguleerd in (de voorstadia van) cervix carcinoom. HLA klasse I moleculen activeren T-cellen door antigeen presentatie en zijn derhalve van belang voor de immunologische surveillance. Om bij te dragen aan de tot nog toe beperkte kennis over de onderliggende moleculaire mechanismen van TAP verlies in cervix carcinoom hebben we TAP expressie, LOH en mogelijke

TAP mutaties onderzocht (**CHAPTER 5**). Om de timing van TAP expressieverandering te kunnen bepalen werden 23 cervix carcinomen en naastliggende premaligne laesies gekleurd met HLA-A-, HLA-B/C-, β_2-microglobuline-, TAP1- en TAP2-specifieke monoclonale antilichamen. TAP1 werd niet gedetecteerd in 10 van de 23 cervix carcinomen en 5 van de 10 naastliggende CIN laesies. Alle laesies met lage TAP expressie hadden ook veranderde HLA klasse I expressie. Om tumor- en normale cellen te kunnen onderscheiden werden de cervix carcinoom samples gesorteerd door middel van "flow-cytometry" en vervolgens geanalyseerd voor LOH met markers in het TAP gebied op chromosoom 6p21.3. LOH werd gevonden in 6 van de 10 laesies met TAP verlies. Aansluitend werd een mutatie-analyse op deze monsters uitgevoerd. In 2 gevallen detecteerden we een polymorfisme in de "5'-untranslated region" van het TAP1 gen. Er werden geen mutaties aangetoond. Dit onderzoek laat zien dat er veranderde TAP expressie is in een aanzienlijk deel van de cervix carcinomen. Het onderliggende mechanisme lijkt LOH in het TAP gebied te zijn, hetgeen niet gepaard gaat met een mutatie. In alle gevallen met lage TAP expressie werd ook HLA klasse I verlies geconstateerd, een bevinding die eerdere rapporten over een sterke associatie tussen TAP aberraties en verlies van HLA klasse I onderschrijft.

Humaan papillomavirus (HPV) is een voorwaarde voor de ontwikkeling van cervix carcinoom. Het is gebleken dat multipele HPV infecties vaak vóórkomen in premaligne stadia. Recent werden ook in invasieve cervix tumoren multipele HPV infecties vastgesteld. Derhalve onderzochten we de significantie van multipele HPV infecties door het bestuderen van de prevalenties van deze in cervix carcinoom in een laag-risico (Nederlandse) en een hoog-risico (Surinaamse) populatie en de correlatie van HPV infectie met tumorcel aneuploïdie (**CHAPTER 6**). SPF_{10} LiPA werd gebruikt voor HPV detectie en typering in 96 Nederlandse en 95 Surinaamse cervix carcinomen. Vervolgens werden laesies met gecombineerde HPV 16/18 infecties met behulp van flow cytometry gescheiden in de diploïde en aneuploïde tumorcel fracties. Deze fracties werden HPV getypeerd door HPV 16- en HPV 18-specifieke PCRs. HPV integratie werd onderzocht op deze gesorteerde cervix carcinoomcellen. Fluorescent in situ hybridisatie (FISH) op paraffine materiaal werd gebruikt voor gelijktijdige detectie van HPV 16 en 18 genotypen en uitgevoerd op de gesorteerde samples.

Multipele HPV infecties waren aanwezig in 13.8% Nederlandse en 22.1% Surinaamse HPV positieve cervix carcinomen. Drie tumoren hadden een HPV 16 en HPV 18 co-infectie: in twee tumoren werden de geïntegreerde HPV copieën van óf HPV 16 óf HPV 18 gedetecteerd in de aneuploïde fractie. In de derde tumor waren zowel HPV 16 als HPV 18 alleen episomaal aanwezig. Deze resultaten tonen aan dat multipele HPV infecties vóórkomen in cervix carcinomen van zowel hoog- als laag-risico populaties. Meerdere HPV typen kunnen in episomale vorm voorkomen in zowel de diploïde als de aneuploïde tumorcellen, maar geïntegreerd HPV DNA werd alleen in aneuploïde tumorcel subpopulaties gedetecteerd.

Conclusies die werden getrokken en hypothesen die werden geformuleerd zijn in **CHAPTER 7** in het perspectief van recente literatuur geplaatst.

List of Publications

1. Vermeulen CFW, Jordanova ES, Zomerdijk-Nooijen YA, Ter Haar NT, Peters AAW, Fleuren GJ. Frequent HLA Class I Loss is an Early Event in Cervical Carcinogenesis. Human Immunology 2005 Nov; 66(11): 1167-73.
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3. Vermeulen CFW, Grünberg A, Peters AAW, Van der Linden-Narain IBS, Vrede MA, Krul EJT, Dekker FW, Fleuren GJ. Ethnic Patterns of Cytological Abnormalities in Cervical Smears in Suriname, a High-Risk Area for Cervical Cancer. Acta Cytologica 2006 Nov-Dec; 50(6): 621-6.
4. Vermeulen CFW, Boon ME, Grünberg A, Van der Linden-Narain IBS, Vrede MA, Dekker FW, Peters AAW, Fleuren GJ. Decreased Prevalence of Dysplasia in High-Risk Population Immigrants in a Low-Risk Area for Cervical Cancer. Int J Gynecol Cancer 2007 May-Jun; 17(3): 646-50.
5. Vermeulen CFW, Jordanova ES, Ter Haar NT, Kolkman-Uljee S, Miranda NF, Ferrone S, Peters AAW, Fleuren GJ. Expression and genetic analysis of transporter associated with antigen processing in cervical carcinoma. Gynecological Oncology 2007 Jun; 105(3): 393-9.
6. Vermeulen CFW, Jordanova ES, Szuhai K, Kolkman-Uljee S, Vrede MA, Peters AAW, Schuuring EMD, Fleuren GJ. Physical Status of Multiple Human Papillomavirus Genotypes in Flow-Sorted Cervical Cancer Cells. Cancer Genetics and Cytogenetics 2007 Jun; 175(2): 132-7.

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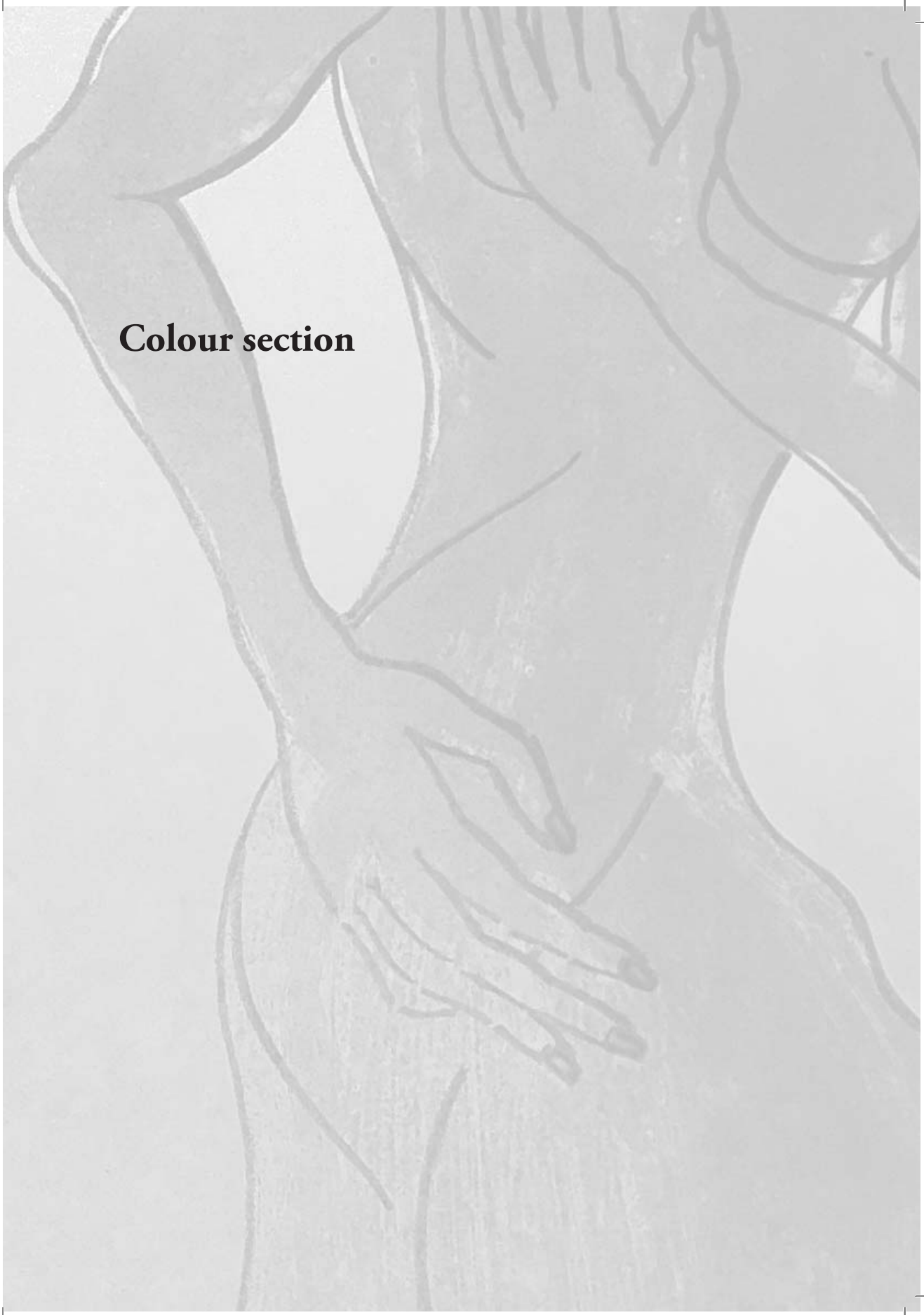
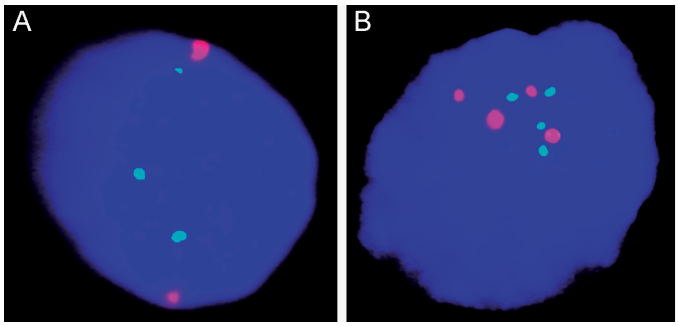
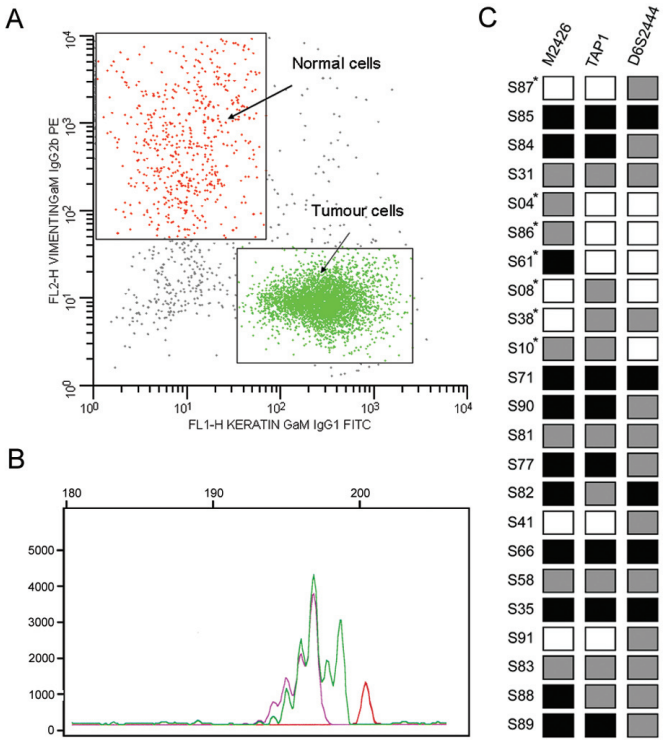
Colour section





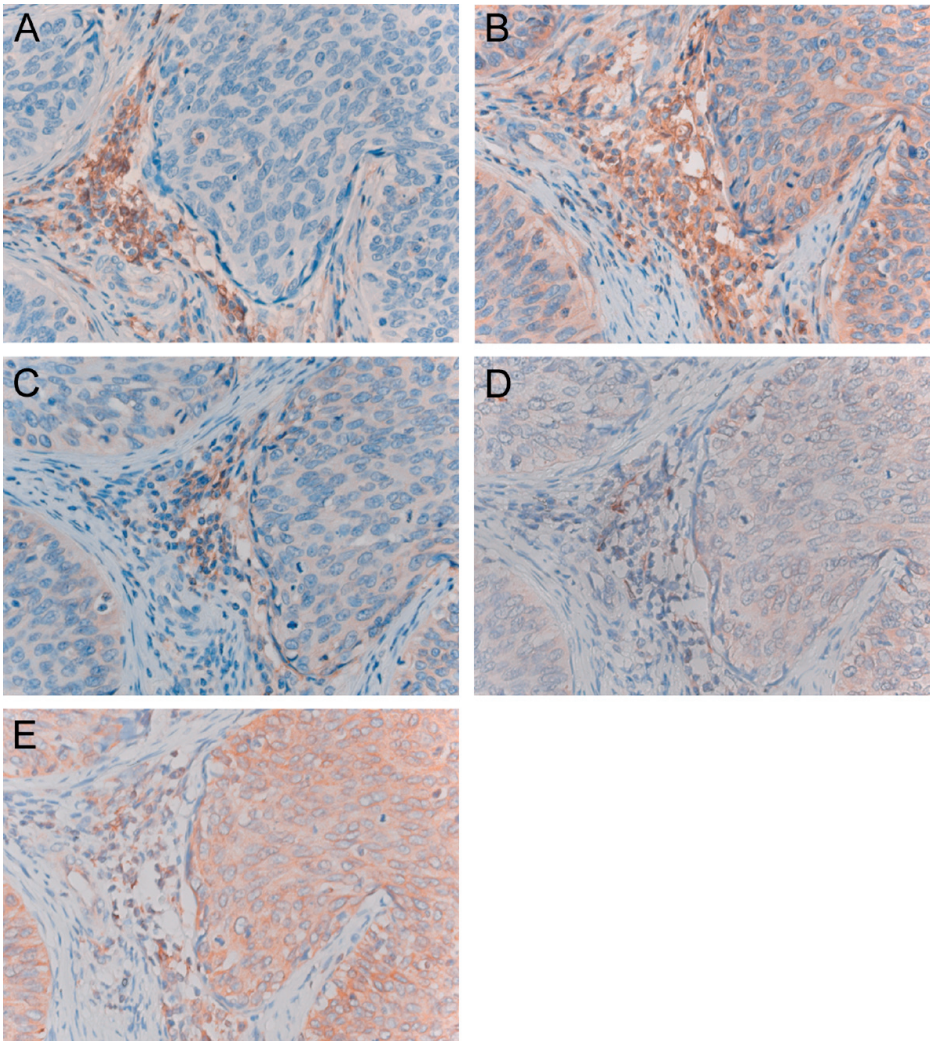
Chapter 4, FIGURE 2

Examples of the FISH results. (**A**) In case S77, FISH showed two centromeric signals and three signals of the whole HLA class I region in 16% of the nuclei, suggesting a possible duplication and translocation. (**B**) Example of aneusomy 6, found in most of the cases.



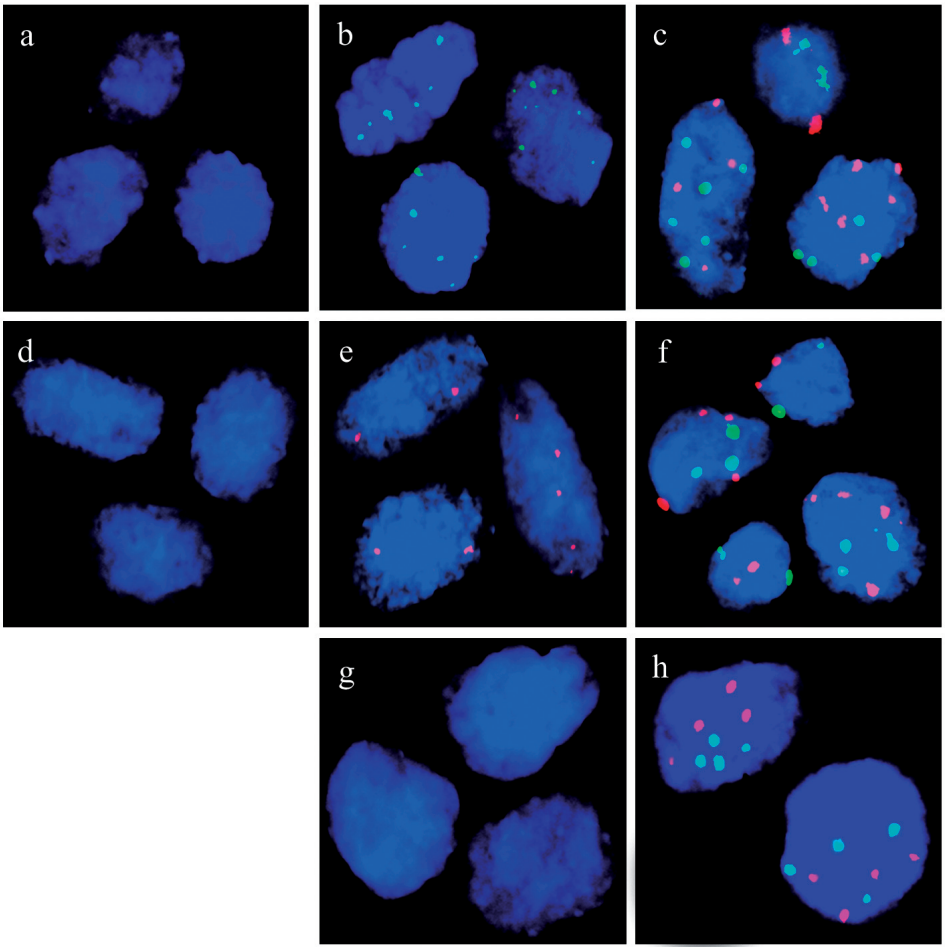
Chapter 5, FIGURE 3

LOH analysis results. (**A**) A representative example of flow-sorting data of a cervical cancer sample (S41). The keratin positive (tumour, FITC-labelled) cells and the vimentin positive (normal, PE-labelled)) cells were flow-sorted and used in further analyses. (**B**) LOH results (S87) at marker TAP1 for tumour (pink, one peak) and normal (green, two peaks) sorted cell fractions (S87). A size marker is depicted in red. (**C**) The complete LOH data of the three microsatellite markers used per tumour sample, represented as ROH (black squares); LOH (white squares) and not informative (grey squares). The same order of samples is used as in **FIGURE 1**.



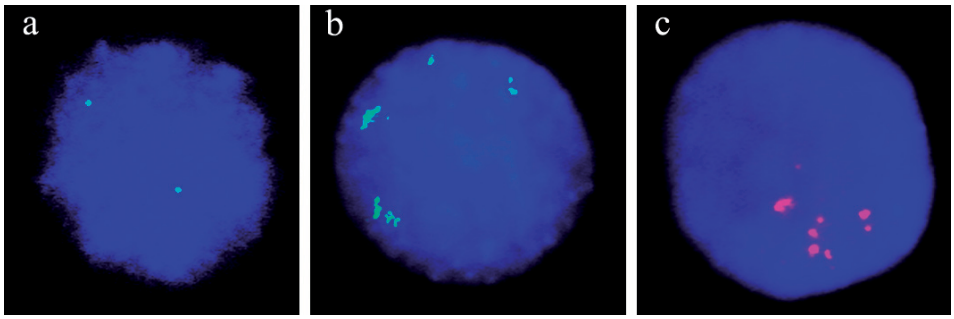
Chapter 5, FIGURE 2

Immunohistochemical staining of a cervical carcinoma lesion (sample S87). Detail (400x magnification) of the same group of tumour cells, stained with TAP1 (negative) (**A**); TAP2 (positive) (**B**); HLA-A (**C**) and HLA-B/C (**D**) (weak cytoplasm, negative membrane); β2M (positive cytoplasm) (**E**).



Chapter 6, FIGURE 1

Interphase FISH on flow-sorted cervical carcinoma cells of the HPV 16/18 positive cases. Case 1 (**A**): the diploid cells are negative for HPV; (**B**) The aneuploid tumour cells show punctate signals for HPV 16 (green); (**C**) Control centromere 1 (red) and centromere 6 (green) signals. Case 2 (**D**): the diploid tumour cell fraction is negative for HPV; (**E**) The aneuploid tumour cells show punctate signals for HPV 18 (red); (**F**) Control centromere 1 (red) and centromere 6 (green) signals. Case 3 (**G**): the aneuploid tumour cell fraction is negative for HPV; (**H**) Control centromere 1 (red) and centromere 6 (green) signals.



Chapter 6, FIGURE 2

Interphase FISH on flow-sorted cervical cancer cell lines. (**A**) SiHa: 2 copies of HPV 16 are visible in green; (**B**) CaSki: multiple copies of HPV 16 are visible in green; (**C**) HeLa: multiple copies of HPV 18 are visible in red.