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POPULATION BASED STUDIES FROM THE VÄSTERBOTTEN COUNTY IN NORTHERN SWEDEN

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To my beloved daughter Amanda
and my wife Noushin

TABLE OF CONTENTS Page

SUMMARY

Infection with human papillomaviruses (HPV) types 16 and 18 is the major cause of cervical neoplasia. Although a high proportion of cervical cancers (CXCA) harbor HPV genomes, only a small number of women infected with high-risk papillomaviruses develop cervical tumors, suggesting that other environmental and/or genetic factors contribute to cervical carcinogenesis. Several studies have identified genes encoding human leukocyte antigens (HLA) associated with cervical intraepithelial neoplasia (CIN) and CXCA.

Many genes encoding the products involved in immune responses are clustered within the human major histocompatibility complex (MHC) on the short arm of chromosome 6 (6p21.3). The human MHC extends over 3500 kilobases and comprises more than 200 genes with known and unknown functions.

The HLA class I genes encodes cell surface glycoproteins (HLA-A, -B, -C) that associates in the endoplasmic reticulum with ß2-microglubulin and peptides derived from endogenously processed antigens. HLA class II genes encode cell surface glycoproteins, which bind to peptides that originate mainly from exogenous antigens processed through the endosomal/lysosomal pathway.

In addition to HLA class I and class II molecules, genes that encode the cytokines, tumor necrosis factor (TNF) α and β are located in the class III region. The MHC class I chain related genes (MICA/B) located in the centromeric end of the HLA class I region, have recently been in focus for it can function as a ligand for γ/δ T cells and NK cell receptors. MICA protein is mainly expressed by epithelial cells and its interaction with NK cells and γ/δ T cells might have a role in the pathogenesis of CXCA.

The aim of this thesis was to investigate the association of candidate HLA genes with CIN and CXCA. Several different genotyping methods were used to study the polymorphic HLA genes in two different patient groups. Patients and controls from a cohort of Västerbotten were included in this population-based study. Candidate genes in the HLA class I, class II and class III region were analyzed and their association with CXCA and susceptibility to HPV infection was measured using appropriate statistical methods. The strongest association of HLA genes with CXCA was found in the HLA class II locus. The relative risks of CXCA among DR15 and DQ6 (DQB1*0602) positive patients were 3.73 and 4.33, corresponding to population attributable proportions of 27.9% and 30.8%, respectively. MICA was not associated with either CIN or CXCA. The polymorphism of the TNFA gene was associated with susceptibility to HPV 16 infection and increased the risk for CIN and CXCA in patients with the DR15-DQ6 haplotype.

PUBLICATIONS

This thesis is based on the following articles that will be referred to in the text by their Roman numerals.

I. MICA gene polymorphism and the risk to develop cervical intraepithelial neoplasia.

Ghaderi M, Hjelmstrom P, Hallmans G, Wiklund F, Lenner P, Dillner J, Sanjeevi CB.

Hum Immunol. 1999 Oct; 60 (10): 970-3.

II. Tumor necrosis factor a-11 and DR15-DQ6 (B*0602) haplotype increase the risk for cervical intraepithelial neoplasia in human papillomavirus 16 seropositive women in Northern Sweden.

Ghaderi M, Nikitina L, Peacock CS, Hjelmstrom P, Hallmans G, Wiklund F, Lenner P, Blackwell JM, Dillner J, Sanjeevi CB.

Cancer Epidemiol Biomarkers Prev. 2000 Oct; 9 (10): 1067-70.

III. Risk of invasive cervical cancer associated with polymorphic HLA DR/DQ haplotypes.

Ghaderi M, Wallin KL, Wiklund F, Nikitina Zake L, Hallmans G, Lenner P, Dillner J, Sanjeevi CB., Submitted.

IV. Tumor necrosis factor A and MHC class I chain-related gene A (MICA) polymorphisms in Swedish patients with cervical cancer.

Ghaderi M, Nikitina Zake L, Wallin KL, Wiklund F, Hallmans G, Lenner P, Dillner J, Sanjeevi CB.

In press: Hum Immunol.

ABBREVIATIONS

Ag	antigen
BD	Behcet's disease
bp	base pair
β2M	β2-microglobulin
CD	clusters of differentiation
Chr	chromosome
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CLIP	the class II-associated invariant chain peptide
CTL	cytotoxic T lymphocyte
CXCA	cervical cancer
DNA	deoxyribonucleic acid
E	early
ELISA	enzyme-linked immunosorbent assay
HIV	human immunodeficiency virus
HSP	heat shock protein
HLA	human leukocyte antigen
HPV	human papillomavirus
IARC	International Agency for Research on Cancer
Ig	immunoglobulin
Kb	kilobase
KIR	killer cell immunoglobulin-like receptor
L	late
LD	linkage disequilibrium
mAB	monoclonal antibody
MHC	major histocompatibility complex
MIC	MHC class I chain-related gene
NK	natural killer cell
NKG2D	natural killer cell receptor
OR	odds ratio
Pap	Papanicolaou (cervical smear test)
Pc	corrected *p*-value
PCR	polymerase chain reaction
p53	tumor suppressor protein 53
Rb	retinoblastoma
RR	relative risk

TAP	transporters associated with antigen processing
TCR	T cell receptor
TM	transmembrane
TNF	tumor necrosis factor
VLP	virus-like particle

INTRODUCTION

Overview

The increasing evidence that susceptibility to certain cancer types is observed more often in immunocompromised individuals, has led to the hypothesis that immune responses control development of tumors. In addition, understanding of the molecular processes that lead to progression of tumors has shed light on identifying new tumor antigens as targets to be aimed by the immune system.

In the case of cervical carcinoma, E6 and E7 oncoproteins of the human papillomavirus (HPV) types 16 and 18 are suitable targets for immunotherapy and preventive strategies since they are continuously required for maintenance of cervical epithelial transformation. Tumor specific immunity, mediated by helper T lymphocytes is now considered to be crucial for efficient eradication of several tumors. The combined knowledge of the molecular mechanisms of class II human leukocyte antigen (HLA) interactions with viral peptides will hopefully provide new insight to trigger tumor specific responses.

Cancer

Cancer arises when the balance between the number of new cells produced and the number of mature cells that die is disrupted. Cancer cells do not necessarily grow faster than normal cells, but they persist longer or divide more frequently during their lifetime. Consequently, cancer cells accumulate, competing with normal, healthy tissue for survival.

In healthy organs, balanced numbers of cells are produced and die each day. In a benign or malignant tumor, several genes regulate processes that are abnormal. As cancerous malignant tumors grow, regulatory genes are damaged, leading to production of more cells or reduction in normal cell death rate.

In general, cancers are divided into four major groups, assorted according to the body tissues in which they arise. All types can spread to other types of tissues of the body, while keeping their original cellular characteristics.

***1) Carcinomas*:** tumors that begin in epithelial tissue (as cervical carcinoma).
2) Sarcomas: tumors originating in the connective tissues, predominantly the muscles, skeleton and cartilage.
3) Lymphomas: tumors in the lymphatic system.
***4) Leukemias*:** cancers of the tissue forming the blood cells.

Historical aspects and etiology of cervical cancer

Venereal warts were known since Roman era. The Roman word for wart is verruca, which means little hill. The Romans suspected that genital warts were sexually transmitted but paid little attention to their cause.

In 1907, Cioffo demonstrated that the human common skin wart contained an infectious agent, which could induce wart into the skin of the volunteers (Cioffo 1907). The theory that warts were transmissible was not generally accepted until in 1949 Strauss *et al* visualized virus particles extracted from warts using electron microscopy (Strauss et al. 1949). The suggestion that genital condylomas were sexually transmitted came when studies performed by Teokharov, Oriel and von Krogh proofed a link between venereal warts and sexual activity (Oriel 1971; Teokharov 1969; von Krogh 1975).

The assumption that cervical cancer (CXCA) was caused by a sexually transmitted agent was not considered for 30 years ago, until studies by zur Hausen *et al* demonstrated a potential role of human papillomavirus (HPV) in development of cervical tumors (zur Hausen et al. 1974a; zur Hausen et al. 1974b).

Cervical cancer is currently the third most common type of cancer among women worldwide (Parkin et al. 1999) and the second common cancer among women in third world (Pisani et al. 1997). More than 470,000 new cases are diagnosed each year, predominantly among the disadvantaged regions, in both developing and industrialized countries. The incidence of CXCA in developing countries is up to 30 cases per 100,000 women where the diagnosis is usually made in advanced stages. Employing screening programs based on the Papanicolaou (Pap) smear and pelvic examination has led to a steep decline in incidence and deaths from CXCA. However despite routine cervical screening of women in Sweden, 500 new cases of invasive cervical cancer are diagnosed each year and 3500 patients with serious CIN are treated annually (Rylander 1995).

CXCA occur in a wide range of ages, mainly from mean age of forties to fifties. Cervical intraepithelial neoplasia (CIN) on the other hand, which is a precursor of CXCA, is classified histologically into three classes (CIN I-CIN III), based on its severity. The level of cellular changes is determined by histopathology and microscopy of Pap smears. CIN III is often called as cervical carcinoma *in situ*. The peak incidence of cervical cancer *in situ* occurs at younger age as compared to invasive type. *In situ* cancer is most common around age thirty (Magnusson 2000).

Both CXCA and precursor lesions have now been firmly associated with high-risk HPV infection (Sincock et al. 1992; Wallin et al. 1999; zur Hausen 2000). CXCA and CIN are essentially caused by sexually transmitted and persistent HPV infections transmitted through genital-to-genital contact. Certain strains of HPV are associated with different risk levels of the transformation into cervical tumors. The majority of invasive cervical cancers contain HPV DNA from high-risk HPV strains 16 or 18 (Munoz 2000). HPV 16, 18, and 31 are constantly associated with moderate to severe cases of cervical dysplasia and, less associated, with invasive cancers of the vulva, penis, and anus. It has been demonstrated that even minimal amounts of HPV DNA is associated with an increased risk in the development of cervical cancer (Munoz et al. 1992). HPV DNA is found in up to 100% of cervical tumors (Figure 1), while in cervical tissue of healthy women the figure is typically below 10% (Wallin et al. 1999).

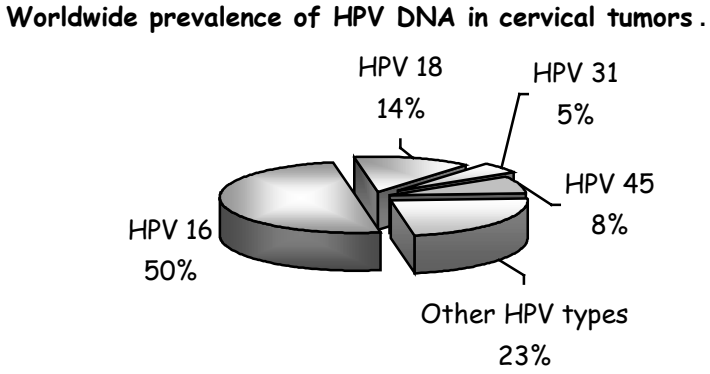

Figure 1: Source (Bosch et al. 1995)**.**

HPV and carcinogenesis

Human papillomaviruses belong to Papovaviridae family together with Polyomaviruses (Matthews 1979). HPV is a non-enveloped, circular double stranded and relatively small DNA virus with 7900 bp genome. Papillomaviruses have been identified in most vertebrates. There exist more than 85 types of sequenced HPVs (zur Hausen 1999b). HPVs have a specific tropism for squamous and glandular epithelial cells, and the stage of productive infection is related to the stages of the cell differentiation. To establish an infection, viruses have to infect basal stem cells of mucosal epithelium. In most infections the virus stays in an episomal state. In invasive cervical tumors, the viral DNA is integrated into the host genome while in benign tumors the integration into the host genome is a rare event (Klaes et al. 1999).

The HPV genome consists of three functional regions. The early (E) region consists of open reading frames, the products of which controls viral replication, transcription, and cellular transformation. It also encodes for the E6 and E7 oncoproteins. The late (L) region encodes for the structural proteins, L1, the major and L2, the minor capsid proteins. The so called "long control region" contains transcription enhancer genes and promotor elements. To date, the main vaccine strategy is to target products of E6, E7, L1 and L2 genes.

Three viral oncogenes have been identified in high-risk HPVs. E5 expression does not lead to immortalization of human cells; in addition, the gene is frequently deleted in cervical carcinoma lines (Schwarz et al. 1985). E5 may be required for early events in natural HPV infections (zur Hausen 1999a).

The E6 and E7 genes in contrast mediate a key role in transformation of the cervical epithelia (zur Hausen 1999b). The E6 oncoprotein of the high-risk HPV types combines with the tumor suppressor protein p53 and disrupts its tumor suppressor properties (Scheffner et al. 1990). The E7 protein encoded by high-risk HPV types shows high-affinity association with the retinoblastoma tumor suppressor, pRb. The E7 protein associates also with other cellular factors known to function in cell cycle regulation (Munger et al. 1989) (Figure 2).

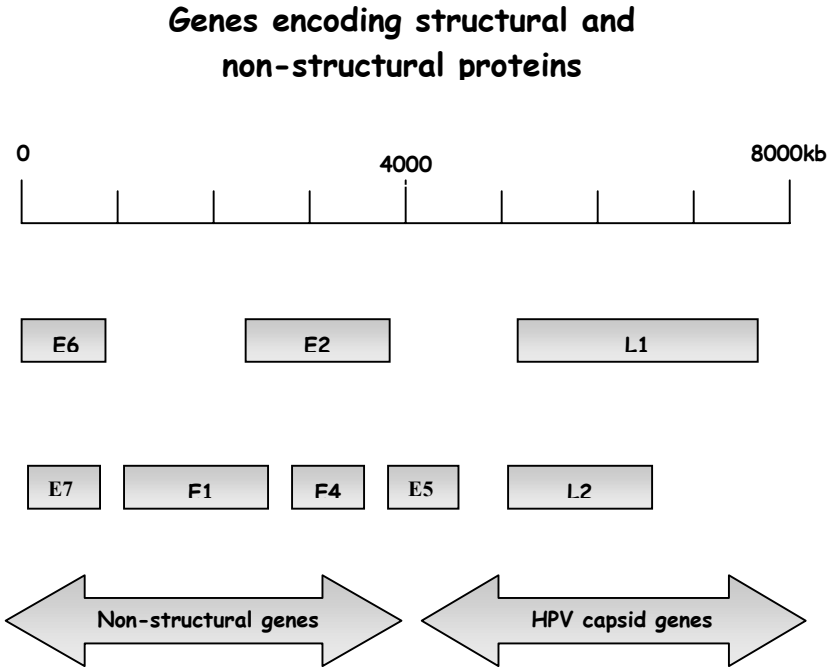

Figure 2: Simplified linear map of a HPV 16 genome. HPV genome is double-stranded and circular.

Specific HPV viral oncogenes are necessary but not sufficient for cell immortalization, for malignant conversion and for the maintenance of the malignant state. Thus, although the E6 and E7 oncogenes are required for HPV-mediated cell immortalization and for the malignant phenotype of cervical carcinoma cells, their expression is clearly not sufficient to immortalize and transform cells. Host cell genes interfering with their function or expression have to be modified in the course of transition to a different growth pattern (zur Hausen 1989). It appears also that not all women infected with HPV develop tumor, indicating that host genetic factors play an important role in the clearance of HPV infection, genetic host factors such as HLA genes could be important in development of cervical cancer. Both family studies and studies on monozygotic twin's

point strongly to a genetic link to cervical cancer (Ahlbom et al. 1997; Magnusson et al. 1999).

Epidemiological studies have shown that the total number (lifetime) of sexual partners and the age at first coitus are important risk factors for development cervical cancer (Parazzini et al. 1998). A correlation between number of lifetime sexual partners and increased risk for HPV infection is shown below (Table 1).

Table 1: Presence of HPV DNA in relation to sexual history.

No. of lifetime partners	No. of women	Positive women (%)	OR	CI (95%)
0-1	74	5	1.0	-
2-5	252	32	8.1	2.9-31.6
5+	186	34	9.1	3.2-36.0

Source (Kjellberg et al. 1999)**.**

THE IMMUNE SYSTEM

Diversity in the immune system

Diversity of the immune system is a hallmark in vertebrates. T- and B-lymphocytes with millions of distinct specificities function in harmony with large diversity of cytokines, chemokines and antigen presenting cells (APC's) to defend vertebrates against infections. Different pathogens are handled by different immune responses, varying from cellular to humoral responses. At the same time, unwanted immune responses against self-proteins are avoided. However due to the polymorphism of the vertebrate major histocompatibility complex (MHC) genes, different individuals in a population respond differently to identical antigens.

MHC polymorphism is in fact a result of host-pathogen co-evolution in which MHC molecules play a key function in cellular immune responses. When a pathogen infects a host cell, the proteins of the pathogen are degraded intracellularly, and a subset of different peptides are loaded onto the groove of MHC molecules, which are transported to the cell surface. Once the MHC-peptide complex is present on the cell surface, T lymphocytes can mount an immune response against pathogens.

Due to the high population diversity of MHC molecules, different individuals will mount an immune response against different subsets of the peptides of any particular pathogen. Pathogens that escape from presentation of an individuals MHC may not to be able to escape from presentation in another host with different MHC molecules. This explains in part the MHC-related susceptibility in infectious diseases and why some autoimmune disorders in human are linked to the genes located in the HLA region.

Immunity to HPV infection

Two main arms of the immune response play an important role in the natural clearance of HPV infection, innate and adaptive immunity. Innate immunity consists of a quickly induced, non-specific response, which does not result in immunological memory. The innate immune system is localized at epithelial borders and is stimulated via several immunomodulatory cytokines and cellular effectors including monocytes, macrophages, natural killer (NK) and antigen presenting cells (APC). The innate immune system is likely to play an important first line of defense in the control of papillomavirus infections. It is triggered by evidence of cellular distress (for example from a viral infection) and leads to the destruction of the affected cells and the pathogen. The adaptive immune response arises from a breach of the innate immune response resulting in the generation of antigen specific effector cells and their products that specifically target the pathogen or pathogen infected cell, as well as memory cells that will prevent or limit subsequent infection with the same organism. It is now presumed that activation of the innate immune system stimulates the adaptive immune responses.

Since HPV infections are not systemic and remain at the site of initial infection, the first line of defense would be at the mucosal surface via the innate immune system. Some insight into these early events may be derived from studies of regression of genital warts, which is associated with an increase in infiltration of macrophages, NK and CD4+ T cells (Coleman et al. 1994).

Generally HPV infections are characterized by a very high rate of spontaneous clearance suggesting that immune responses play the essential role in the control of infection. Follow-up studies of women with a HPV DNA positive estimate 70% clearance during a 12-month period (Evander et al. 1995; Hildesheim et al. 1994). It is women with persistent HPV type-specific infection that are in risk zone to develop invasive cervical cancer (Wallin et al. 1999). Several lines of evidence

suggest also that the immune responses play an important role in the control of HPV infection. HPV antibodies seem to have little protective effect, and cell-mediated immunity seems to play a more important role. Most convincing are the observations that HPV-associated cancers occur more commonly among those with cell-mediated immune deficiencies, such as patients with renal transplants (Dyall-Smith et al. 1991).

The HLA system

The human MHC is known as human leukocyte antigen system, which is located on the short arm of chromosome 6 (6p.21.3), is divided into three gene regions (class I, class II, and class III). The HLA complex contains over 200 genes, more than 40 of which encode leukocyte antigens **(**Figure 3). The nucleotide diversity within human MHC is unusually high (8-10%) (Gaudieri et al. 2000), compare to the rest of the genome where nucleotide diversity is estimated between 0.08% and 0.2% (Horton et al. 1998). A variety of genes with known and unknown immune mechanisms are encoded within the human MHC between the class I and class II genes. Genes encoding complement subunits C4, C2 and Factor B as well as genes that encode the pro-inflammatory cytokine tumor necrosis factor (TNFα, cachectin), TNFβ (Lymphotoxin) and genes encoding adrenal 21-hydroxylase enzyme (CYP 21A and CYP 21B) are located in the class III region.

The main function of the MHC molecules is to present antigen. *Endogenous* antigens are processed in cytoplasm to peptides and loaded by peptide transporters associated with antigen processing (TAP) on class I MHC molecules in the endoplasmic reticulum. *Exogenous* antigens are engulfed by the cell, clipped in endosome/endocytic compartments to peptides, sent to endosomal membrane to dock with fully synthesized MHC class II-Invariant chain (CLIP) complexes. The Invariant chain is degraded by HLA-DM and HLA-DO, which control peptide loading in acidic compartment of the endocytic pathway. Peptides fill up the cleft, stabilize the MHC class II-Ag complex. MHC class II molecules without any antigen are fragile and dissociate in the acidic compartment of the endosome (Kropshofer et al. 1997a; Kropshofer et al. 1997b). Because the pathways for antigen processing differ, the same protein will be processed differently for MHC class I presentation versus MHC class II presentation. Thus, different antigens stimulate CD8+ T cells versus those that stimulate CD4+ T cells. This process, coupled with the fact that T cells only bind MHC molecules used for their thymic selection, is called MHC restriction (Doherty and Zinkernagel 1975).

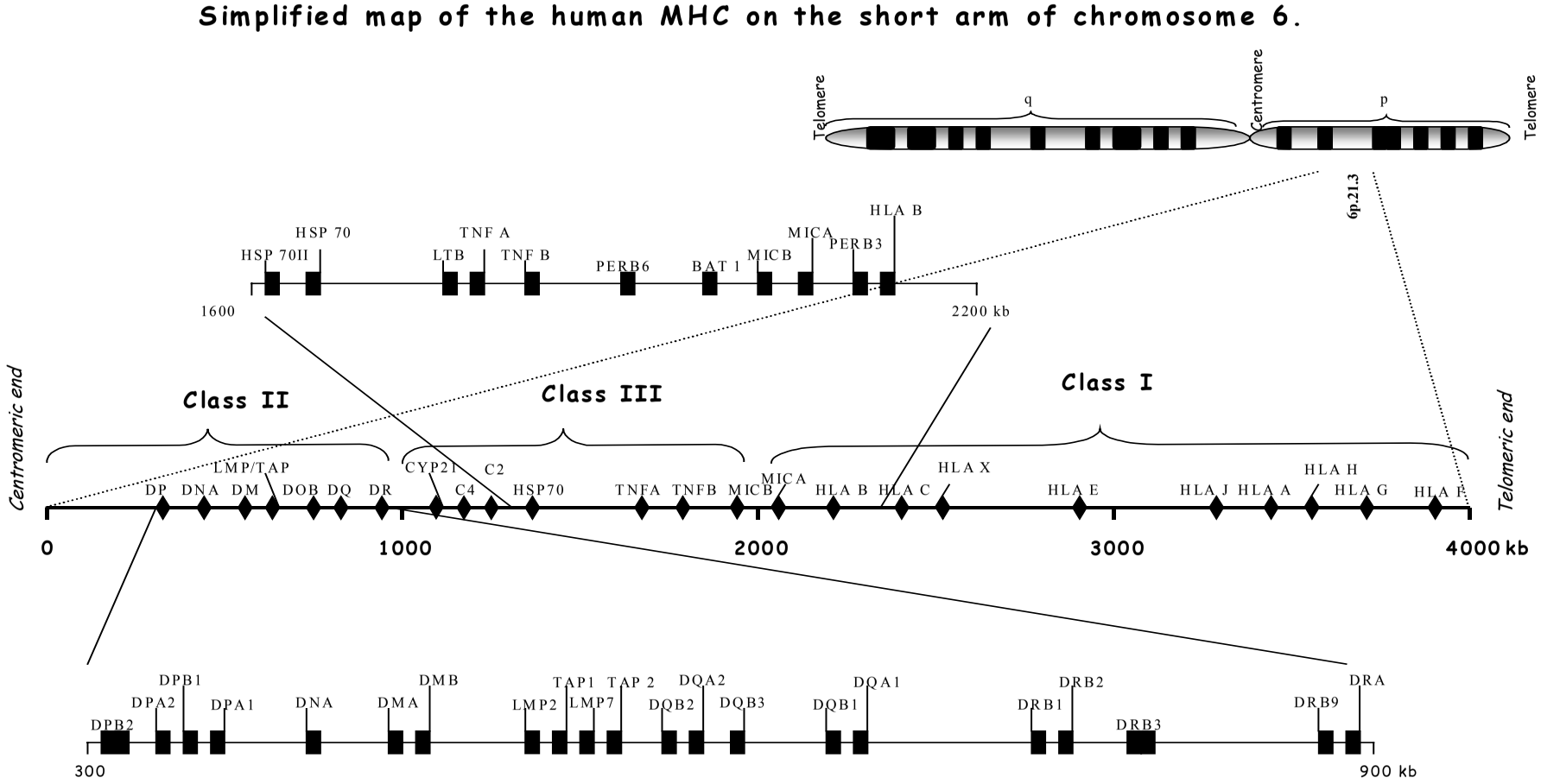

Figure 3: The human MHC contains over 200 genes, more than 40 of which encode for HLA.

Many of the HLA genes appear together in a higher frequency than expected through random association, a phenomenon referred as linkage disequilibrium (LD). For instance, HLA-A1 is commonly associated with B8 and DR3 and A2 with B7 and DR2, presumably because the combination confers some selective advantage. However, genes in the MHC region could be in linkage with genes in vicinity to the MHC. A good example of this is the neurological disorder, narcolepsy. Patients with narcolepsy have a defective gene coding for the receptor (HCRTR2) of neuropeptides called hypocretins (produced in the lateral hypothalamus) (Lin et al. 1999). The mutated HCRTR2 receptor gene is located outside the MHC region close to HLA-DQB1*0602-DQA1*0102 (DQ6). Because of linkage between these three genes, a higher frequency of HLA-DQ6 haplotype is observed among narcolepsy patients than anticipated through random combinations in the population (Matsuki et al. 1992). Nevertheless, contribution of HLA genes to narcolepsy is not excluded since recent studies suggests that human narcolepsy is an autoimmune disorder associated with the destruction of a small number of hypothalamic neurons containing the peptide hypocretins in which the predisposed HLA molecules could be involved.

Modulation of HLA expression in cervical tumors

The extent and the type of T cell activation is depended critically on the expression of MHC. DNA viruses have developed a number of strategies to interfere with MHC expression or function so as to disturb antigen recognition (McFadden and Kane 1994). Down-regulation of class I HLA and up-regulation of class II HLA molecules in cervical neoplasia have been reported in several studies (Bontkes et al. 1998a; Glew et al. 1993; Glew et al. 1992a; Raju et al. 1994).

Down-regulation of HLA class I expression in cervical tumors

Down-regulation of class I MHC molecules is an observable fact in various types of cancers (Ruiz-Cabello et al. 1991). Numerous immunohistochemical studies using monoclonal antibodies have pointed out a comparable result in invasive carcinoma of the cervix (Cromme et al. 1993; Torres et al. 1993). However inconsistent results have been reported on the expression of HLA class I antigens in CIN (Glew et al. 1993; Hilders et al. 1994; Torres et al. 1993). Most low-grade CIN regress and down-regulation or altering of HLA class I expression may be important for persistence and progression of CIN, caused by oncogenic HPV types. Altering HLA class I expression will disturb cytotoxic CD8+ T lymphocyte recognition and it would provide neoplastic cells with a

means to avoid immune surveillance. So far, no link has been found involving class I expression and the detection of DNA from specific HPV genotypes (Connor and Stern 1990; Cromme et al. 1993). Nevertheless, it is known that in certain HPV related cancer types expression of TAP-1 is inhibited which decrease the rate of peptide loading on the HLA class I molecules (Keating et al. 1995; Vambutas et al. 2000).

P53 is degraded through ubiquitin proteolysis when bound to HPV 16 E6 protein (Scheffner et al. 1990). As a consequence, enhancement of TAP expression is indirectly inhibited by HPV 16 E6 oncoprotein because induction of TAP is upregulated by p53 (Zhu et al. 1999). Defective assembly of class I antigens makes them unstable on the cell surface (Elliott 1996). This pathway explains in part why expression of HLA class I antigens are downregulated on cervical tumors.

Up-regulation of HLA class II expression in cervical tumors

Although normal squamous cervical epithelium is HLA class II negative, more than 80% of cervical squamous cell carcinomas show expression of class II HLA antigens, detected by immunohistochemical methods (Glew et al. 1992a). HLA-DR and HLA-DP are more commonly expressed than HLA-DQ and in some cases all are up-regulated. Increasing HLA class II expression is correlated with the severity of dysplasia in cervical pre-malignant CIN lesions (Cromme et al. 1993; Glew et al. 1993). The status of HLA class II expression by epithelial cells is often influenced by cytokines such as IFNγ and TNFα (Coleman and Stanley 1994) however the HLA class II up-regulation in cervical lesions does not appear to be a generalized response to cytokines, since there is no correlation with the extend of leukocyte infiltration. Up-regulation of class II HLA expression has been described in a variety of different tumor types and can have prognostic significance (Garrido et al. 1993). Although other markers such as Laminin-5 would give more useful hints to study progression of cervical tumors (Skyldberg et al. 1999). The up-regulation of class II HLA expression in the primary tumors may influence the immune response to HPV infection. Cervical tumor cells are known to co-express several of the accessory molecules necessary for antigen presentation (Glew et al. 1992b), but critical co-stimulatory molecules such as CD28 and B7 may be missing, which may lead to induction of tolerance facilitating tumor growth. In fact, increasing co-stimulation of T lymphocytes through CD28 and CTLA-4 receptors by transfection of an E7 positive tumor with B7 led to in vivo tumor immunity mediated by CTLs (Chen et al. 1992).

HLA polymorphisms in cervical neoplasia

Many allelic forms of HLA class I and class II molecules differ by amino acid residues lining in the peptide-binding groove, enabling them to present different set of peptides to T lymphocytes. If cell-mediated immune responses play an important role in controlling HPV infection, the hereditary HLA haplotypes could result in a different immune response to HPV peptides among individuals. This could explain the identification of susceptibility or protective HLA haplotypes among different populations. Available data on HLA associations and papillomavirus-induced cervical neoplasia indicate the importance of the class II genes (Table 2).

Table 2: Some HLA Associations with CIN and cervical cancer.

Population	HLA studied	CIN/CXCA	Reference
Brazilian	DRB1*1501- DQB1*0602↑	CXCA	(Maciag et al. 2000)
British	DRB1*0401-DQB1*0301↑ DRB1*1101-DQB1*0301↑ DRB1*0101-DQB1*0501↓	CIN	(Odunsi et al. 1996)
Dutch	DRB1*07↑	(HPV 16 infection)	(Bontkes et al. 1998b)
French	DRB1*1401/07↑ DRB1*03↑ DRB1*1301/02↓ DRB1*1301/02-DQA1*0103-DQB1*0603↓	CXCA	(Sastre-Garau et al. 1996)
Hispanic	DRB1*0407-DQB1*0302↑ DRB1*1501-DQB1*0602↑	CIN	(Apple et al. 1994)
Honduran	DQA1*0301 ↑, DR4↑ DQA1*0501↓	CIN/CXCA	(Ferrera et al. 1999)
Japanese	DQw3, DQw1↑	CXCA	(Nawa et al. 1995)
Norwegian	DQA1*0102-DQB1*0604↓ DQA1*0102-DQB1*0602↑	CIN	(Helland et al. 1998)
Swedish	DQA1*0102-DQB1*0602↑ DQA1*0501-DQB1*0301↑	CXCA &CIN	(Ghaderi et al. 2001; Sanjeevi et al. 1996)

Positive association↑ – Negative association↓

This points out the importance of helper T cell immunity in the clearance of HPV infections. However, other hypotheses have been proposed for association of HLA genes with CIN and CXCA. For instance a polymorphism in the promotor region of the class II HLA genes would cause an allele-specific divergent expression of HLA molecules, which lead to defective antigen presentation to stimulate T lymphocytes (Davies and Stauss 1997)

The most frequently reported positive association with CXCA has been that of HLA-DQ3 and HLA-DQ6 (DQA1*0102-DQB1*0602) (Apple et al. 1995; Gregoire et al. 1994; Helland et al. 1994; Helland et al. 1998; Sanjeevi et al. 1996). In our latest study, HLA-DQ6 and HLA-DR15 is

positively associated with CIN and CXCA (Ghaderi et al. 2001). Interestingly our results have been replicated in a population-based study on Norwegian (Helland et al. 1998) and Brazilian women (Maciag et al. 2000). In the other hand association of DR15 and DQ6 was not found significant for patients of Northwest England (Duggan-Keen et al. 1996) and France (Sastre-Garau et al. 1996) but HLA-B7 positive patients had a significantly poorer clinical outcome than the HLA-B7 negative patients. HLA-B7 is in fact in linkage disequilibrium with HLA-DR15-DQ6 as an extended haplotype.

The MIC genes

In addition to the class I and class II MHC molecules, which present peptides to the T cell receptor, several non-classical MHC antigens have been identified that are important in induction of innate immune system. The major histocompatibility complex class I chain-related (MIC) genes, encoding MICA and MICB molecules were first discovered by Bahram et al (Bahram et al. 1994). The MIC gene family (MICA-D) is located in the human MHC region centromeric to the class I genes between the TNF loci and HLA-B genes (figure 3). MICA/B encode cell surface proteins while the rest of MIC family being pseudogenes.

MICA/B are expressed at only minimal levels on normal cells. They are richly present on a wide variety of tumors, particularly of epithelial origin including renal, breast, colon, and lung carcinomas (Groh et al. 1998). Amino acid sequence of the MICA chain suggests that it folds similarly to typical class I chains and may have the capacity to bind peptides or other short ligands (Bahram et al. 1994).

Unlike conventional MHC class I genes, MICA/B are not induced by γ-interferon but are regulated by cell stress, similar to the genes for heat shock protein 70 (hsp70) (Groh et al. 1996). MICA/B do not associate with β2-microglobulin (β_2M) so their expression would not be affected by the loss of β_2M, which occurs in certain tumors. As a result cells deficient for β_2M (e g; Daudi cells) are able to express MIC antigens (Groh et al. 1998).

Down-regulation of class I HLA molecules and induction of stress-induced MICA/B that are minimally expressed on normal tissues, but stimulated by transformation or unfavorable environmental influences, provides an ideal early warning for the immune system to eradicate tumor cells from epithelial origin. In vitro studies have demonstrated that human

NK cells and T cells are able to kill tumors expressing the MICA/B antigens, suggesting that these molecules may be important in host protection against cancer through induction of innate immunity (Cosman et al. 2001; Pende et al. 2001).

Normal epithelial cells inhibit NK mediated cell lysis via inhibitory MHC class-I-specific, killer cell immunoglobulin-like receptors (KIR). Cellular transformation leads to induction of stress proteins including MICA and recognition of MICA by natural killer cell receptor (NKG2D)–DAP10 stimulates the NK cell, engaging the NKG2D-DAP10 complex cytoplasmic domain, which leads to NK cell activation and release of perforin and granzymes. Inhibitory recognition of MHC class I molecules could moderate or tune the activating signal. Maximal NK cell activation occurs when the target cells down-regulate MHC class I molecules as shown in the figure 4.

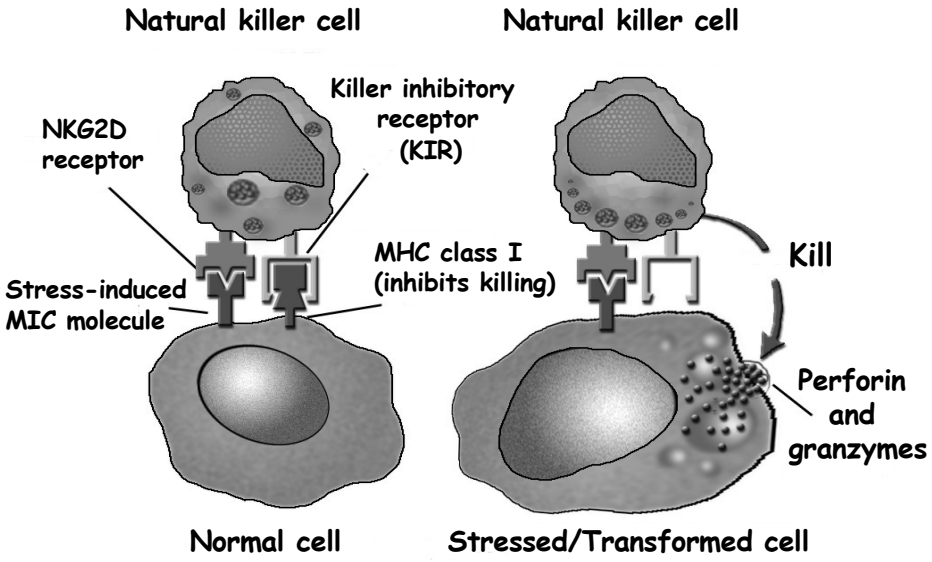

Figure 4: MIC antigen function as ligand for NKG2D receptor.

It was recently revealed that a cytomegalovirus protein UL6 bind to the MICB protein and blocks recognition of MICB through NKG2D receptor (Cosman et al. 2001). Recognition of infected and tumor cells by NK cells, is generally inhibited by MHC class I antigens since MHC class I inhibits target cell death via interaction with killer inhibitory receptors on the NK cells. Because down-regulation of specific HLA antigens is often an early event in cervical neoplasia (Bontkes et al. 1998a), interaction of

NK cells and gamma-delta T cells could play an important role in destruction of transformed cervical tumors. Since the discovery of the MICA gene, more than 45 different alleles of this gene have been reported (Robinson et al. 2001), revealing the extent of the polymorphism within this gene.

The TM region of MICA gene contains a short tandem repeat with five different GCT alleles [$(GCT)_n \rightarrow$ polyalanine)] (Mizuki et al. 1997a). The TM polymorphism of MICA has shown to be associated with several autoimmune disorders in different populations (Cheng et al. 2000; Gambelunghe et al. 1999; Gambelunghe et al. 2000; Kimura et al. 1998; Mizuki et al. 1997c; Park et al. 2001). Mizuki et al studied the localization of the susceptible gene of Behcet disease (BD) using microsatellite markers in 3 different populations. The authors found that only HLA-B51 was significantly associated with BD in all 3 populations. These data suggested that the pathogenic gene of BD is HLA-B51 itself and not other genes found close to HLA-B such as MICA (Mizuki et al. 1997b).

TNFα

The TNF genes are located in the human MHC class III region. TNFα is an inducible cytokine with a broad range of immunoregulatory and proinflammatory effects which promotes inflammation and cellular apoptosis. TNFα inhibits viral replication alone and in synergy with interferon gamma (Kawanishi et al. 1995). TNFα production by activated macrophages and T lymphocytes acts through two distinct receptors, TNFR1 and TNFR2, to affect cellular proliferation, differentiation, survival, and cell death. TNFα is associated with in vivo and in vitro killing of tumor cells. TNFα and TNFß share a common receptor on tumor cells, which are up-regulated by gamma-interferon (Aggarwal et al. 1985). Various interferons have been known to be synergistic with TNF in antitumor effects in vitro. Genetic variation in the TNF gene have been implicated in a variety of human diseases, including cerebral malaria (Knight et al. 1999), and autoimmune diseases such as myasthenia gravis (Hjelmstrom et al. 1998), rheumatoid arthritis (Field et al. 1997), as well as CIN (Ghaderi et al. 2000). Susceptibility to many of these diseases is thought to be due to a link between genetic basis of TNF and the level of TNFα secretion because certain polymorphisms in the promotor region of TNF correlate with the amount of TNFα secretion (Pociot et al. 1993). There are three polymorphic microsatellite markers in the TNF region. The TNFa microsatellite contains CA/GT dinucleotide repeats, has 13

alleles and is located 3.5 kb upstream of the TNFB gene (Nedospasov et al. 1991).

PROPHYLACTIC AND THERAPEUTIC HPV VACCINES

HPV vaccine strategies

Vaccine development against HPV has been limited because of inability to culture the virus. This is caused by viral transcription and replication being dependent on differentiated state of epithelial cells. However, improved understanding of HPV genome and our immune system have led to a more systematic approach to vaccine development.

Three major strategies are being developed for HPV vaccines. The first one is to induce neutralizing antibodies against structural viral capsid proteins to prevent virus entry in epithelial basal keratinocytes. These prophylactic vaccines have been shown to be effective in animal models (Breitburd and Coursaget 1999). The second approach is to stimulate cell-mediated immune responses against viral non-structural proteins to eliminate cells harboring and expressing the viral genomes. Such vaccines should be able to prevent proliferation of infected keratinocytes and to promote regression of developed tumors. However these vaccines would not prevent HPV infection. The E1 and E2 proteins involved in the replication of viral episomal genomes and in the transcription of viral early genes are probably the best target antigens in the first stage of infection. However, the very low levels of expression of E1 and E2 proteins render difficult their detection by the immune system, probably providing the virus with an escape from elimination. Moreover, these two genes are usually knocked out upon integration of the viral genome into cellular chromosomes, an early event in tumor progression. The E6 and E7 oncoproteins are expressed at all stages of tumor progression, from infection to carcinoma development and are the targets chosen so far for therapeutic vaccines in humans. Since virus-like particles (VLPs) proved to be efficient vectors for gene transfer (Touze and Coursaget 1998) and were found to induce cytotoxic T lymphocyte responses a third approach would be to design a DNA vaccine packaged into HPV VLPs or to construct chimeric VLP vaccines presenting epitopes inducing both prophylactic and therapeutic effects.

MHC-based HPV vaccines

Vaccination strategies against tumor-specific peptides produces neutralizing antibodies and may activate antigen specific CTLs. Since

HPV 16 E6 and E7 peptides are maintained in cervical cells and are considered crucial for malignant transformation, vaccination with HPV 16 E6 or E6 peptides may boost tumor specific responses. Peptides encoded by human CTLs in HLA-A2 restricted fashion have been identified for use in vaccinating patients with HPV 16 positive cervical carcinoma (van Driel et al. 1999). In practice, peptides alone are poorly immunogenic (Murakami et al. 1999). To overcome this limitation, they can be altered to increase their immunogenicity by modification of the peptide amino acid sequences, conjugation with other immunostimulatory agents, or administration with adjuvants. Practically, application of peptide-based vaccines is limited by MHC restriction and the necessity of defining the T cell epitopes.

Presentation of antigenic peptides by class II MHC molecules to CD4+ T lymphocytes is critical to the generation of antitumor immunity. So far, few attempts have been performed to design HPV derived peptides based on MHC class II binding motifs. In the mouse model C57BL/6 (H-2^b) two peptides, 60-VYRDGNPYA-68 and 98-GYNKPLCDLL-107 from the HPV E6 protein has recalled a proliferative response in lymph nodes.

A recent phase I trial with peptide-based vaccine derived from HPV 16-E7 (98 amino acids), induced complete regression of neoplastic lesions in 3 of 18 HPV 16 positive women with high-grade cervical and vulvar intraepithelial neoplasia. Virological assays showed that 12 of 18 patients cleared HPV 16 from cervical scrapings by the fourth vaccine injection, but all biopsy samples tested positive for the virus by in situ RNA hybridization after the vaccination (Muderspach et al. 2000). The data establish that a HPV 16 peptide vaccine may have important biological and clinical effects and suggest that future refinements of an HPV vaccine strategy to boost antigen-specific immunity should be explored.

By performing competitive HLA binding assay (Figure 4) with different HPV 16-E7 peptides we know for instance that peptide **44-QAE-PDRAHYNIV-TFC-58** bind with a good affinity to HLA-DQ6.

HPV 16-E7 oncoprotein

1-MHGDTPTLHEYMLDLQPETTDLYCYEQLNDSSEEEDEIDGPAGQAEPD RAHYNIVTFCCKCDSTLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP-98

However if HPV 16-E7 (44-58) peptide is immunogenic, remains to be tested.

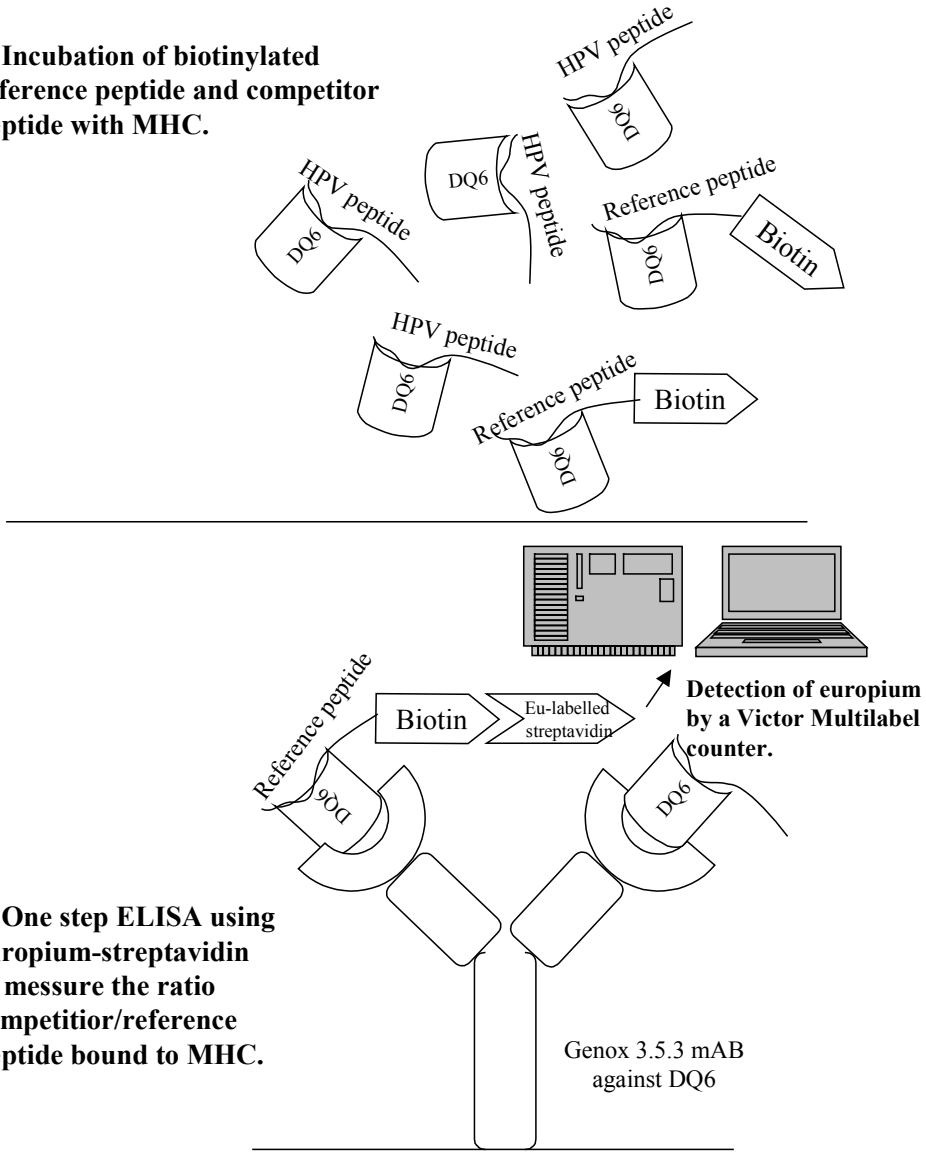

Figure 4: Competetive MHC peptide-binding assay measures the strength of peptide-MHC interactions. Synthetic peptides from HPV compete with the reference peptide in the presence of protease inhibitors. The inhibitory concentration (IC50) of the HPV peptide is acheived when 50% of the reference peptide-MHC interaction is inhibited.

AIMS OF THIS STUDY

I. To investigate whether HLA genes are associated with CIN and if this association correlates with HPV seropositivity.

II. To study whether HLA genes are associated with CXCA and if this association is due to persistent HPV infection.

III. To estimate which candidate HLA genes confer strongest association to HPV infection or cervical neoplasia, identifying at-risk individuals for preventive and therapeutic purposes such as vaccine design based on the risk-HLA molecules.

MATERIALS AND METHODS

Study design, paper I and paper II (CIN study)

Since 1988, blood samples (plasma, erythrocytes and buffy coat) have been collected from individuals participating in a population-based health-promoting project in the Västerbotten County in Northern Sweden. The Västerbotten project was initiated in a northern Swedish county in 1986. Each year, all residents aged 30, 40, 50 and 60 years are invited to participate in a health-promoting project, including the donation of biological specimens for future medical research. The biobank now contains questionnaires and blood samples (plasma, erythrocytes and buffy coat) stored at -80° C, from about 80.000 subjects (Chua et al. 1996; Dillner et al. 1994). Totally 74 CIN patients and 153 controls matched for age and sex were included in this study. Some data lost of TNFa typing excluded a number of patients and controls in paper II.

Study design, paper III and IV (CXCA study)

Linkage between the cytology registry and the Swedish Cancer Registry for the period 1969 to 1995 identified 133 eligible women with invasive cervical cancer diagnosed after the sampling date of a normal smear. Eligible women were defined as Västerbotten resident women who had taken at least one cytologically normal cervical smear with no operative treatment of the cervix. Controls were women in the study base who did not develop cervical cancer before the time-point of diagnosis of a corresponding case, matched for age (same calendar year of birth), time of sampling of a normal smear compared to the pre-diagnostic normal smear of the case and time of sampling of a normal smear taken after diagnosis of cancer in the corresponding case. As genetic markers are not age or calendar time-dependent, the statistical analyses were in this study performed with pooled data without individual matching.

Four case women were excluded because of incorrect entry in the registry. Eleven case women were excluded because of non-invasive cervical neoplasia, leaving 118 cases of invasive cervical cancer. For 12 women, the histopathological specimens were missing. The tissue blocks from 2 cases were inadequate for PCR analysis, leaving 104 cases (85 squamous cell carcinomas and 19 adenocarcinomas) with serial samples available. In case of several prediagnostic smears with normal cytology, the slide taken closest to the diagnosis of the cancer was retrieved. HLA haplotyping could be successfully completed in 85 case women and 120 control women. The mean age of case women was 44.2 years (range from

19.1-74.1) and of the control women 44.1 years (range from 19.5-74.4). Mean length of follow up was 5.6 years for case women (range: 0.5 months-26.2 years) and 6.3 years for control women (range: 0.4-25.4 years).

Ethical approval was obtained from the Institutional Review Board (IRB) of the Umeå University (95-240 and 98-12). As approved by the IRB, informed consent was not obtained but a press conference was held on the 1995-05-23 (resulting in coverage by major regional newspapers) on the study and the fact that informed consent was not obtained. There were no requests from women in the study base to withdraw from the study and all eligible smears could therefore be retrieved, corresponding to a 100% attendance rate.

Extraction of DNA from blood and pap-smears

Genomic DNA was isolated from peripheral blood leukocytes by standard phenol - chloroform DNA extraction. The DNA was dissolved in sterile double-distillated water.

DNA was extracted from archival smears and biopsies as described (Chua and Hjerpe 1995; Chua et al. 1996). All samples were tested for DNA integrity by PCR using human ribosomal gene S14 primers (Chua and Hjerpe 1995; Rhoads et al. 1986) that give150 base pair (bp) amplimers. Samples positive for S14 PCR but negative for HPV DNA were alcohol precipitated and the PCR was repeated.

PCR-based HLA class II genotyping

The polymorphic second exon of the DQA1, DQB1 and DRB1 genes was amplified in a programmable thermal controller with heated lead (PTC-100 MJ Research Inc, USA) using primers specific for each locus as previously described (Erlich and Bugawan 1989). The 25 µl PCR reaction was run in thin-wall PCR tubes containing 100 nanogram of genomic DNA, 0.5 µM of each primer, 200 µM of dNTP's, 1X Ampli-Taq Gold PCR buffer (standard $MgCl_2$ concentration) and 2.5 units of the Ampli-Taq Gold DNA Polymerase (Applied Biosystems, PE Corporation, USA). The PCR mixture was preincubated for 8 minutes at 95°C to activate the Ampli-Taq Gold DNA polymerase and to denaturate the genomic DNA. The polymorphic second exon was amplified in a 30-cycle PCR reaction with denaturation for 1 min at 96°C and annealing with each specific primer at 62°C for DQA1 and 55°C for DQB1 and DRB1, and primer

extension was carried out at 72°C. The PCR products were run in 2% agarose gels and visualised with ethidium bromide (10 µg/ml). The amplified products were manually dotted onto nylon membranes (Amersham, Arlington, USA), under denaturing conditions. The membranes were hybridised with sequence specific oligonucleotides (SSO's), where the 3' end was labelled with (α-32p) dCTP (NEN, MA Boston, USA) and washed under stringent conditions before exposure to X-ray film at –70°C (Sanjeevi et al. 1992). The membranes were stripped of the labelled probe under alkaline conditions and were re-used for probing with other oligonucleotide probes.

Microsatellite typing of TNFA and MICA gene

Analysis of the TNFa microsatellite polymorphism was done as described by Nedospasov and co-workers (Nedospasov et al. 1991). The 5' end of the reverse primer (Pharmacia-Biotech, Uppsala, Sweden) was labeled with HEX fluorescent dye. The PCR fragment sizes were identified in a Perkin-Elmer ABI prism 373 DNA sequencer. An internal size marker (500 TAMRA) was added to each sample to allow accurate determination of allele sizes. We used the Perkin-Elmer software, Genotyper 2.0 to analyze the GeneScan output file. A GeneScan output file from the MICA samples is shown below (Figure 5).

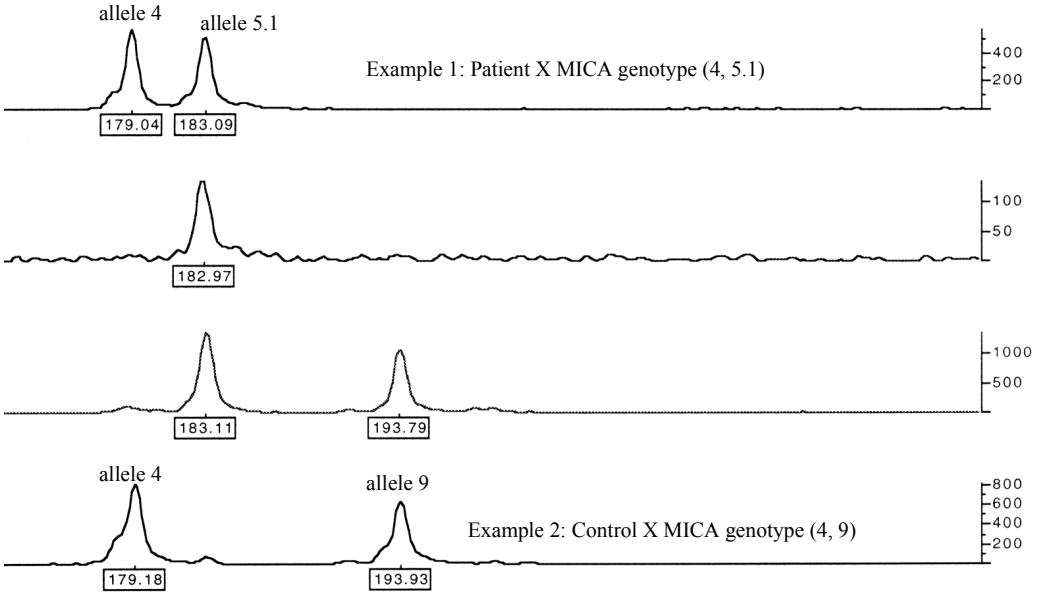

Figure 5: Analysis of MICA alleles by Genotyper (Perkin Elmer, USA) software.

PCR amplification of the MICA microsatellite alleles of exon 5 was carried out using 5' end fluorescent (FAM) labeled primer, MICA-5

reverse (5'-CCTTACCATCTCCAGAAACTGC) and MICA-5 Forward unlabelled primer (5'-CCTTTTTTTAGGGAAAGTGC) (Pharmacia Biotech, Sweden) in an Omnigene thermal cycler (Woodbridge, NJ, USA). The 25 ul PCR reaction containing 1 uM of each primer, 100 ng of genomic DNA, 200 nM of each dNTP (SDS promega, Falkenberg, Sweden), 2 ul (1X) PCR buffer and 1U of the Ampli-Taq gold DNA polymerase (Perkin Elmer, USA) was carried out in a one step PCR reaction in which DNA was denaturated for 10 min at 95C after which in 30 cycles DNA was denaturated for 1 min at 94C, annealed with primers at 55C for 1 min and polymerized at 72C for 1 min with a final cycle of extension at 72C for 15 minutes (Ota et al. 1997).

All PCR products were run on 2% agarose gel (Gibco BRL, Life Technologies, Paisley, Scotland) at 5 volts/cm in TBE buffer 0.5 X. Each well on the gel as loaded with 4ul of the PCR product and 1ul of the loading dye (SDS promega Falkenberg, Sweden). The amplified bands were visualized in gels stained with EtBr (0.25mg/ml agarose).

HPV typing

Presence of HPV DNA and serum antibodies against HPV surface epitopes was done by Keng-Ling Wallin at Joakim Dillners laboratory (Microbiology and Tumor Biology Centrum, Karolinska Institute). The HPV consensus primers MY09 and MY11 (Manos et al. 1989) and GP5+ and GP6+ (de Roda Husman et al. 1995) were used in nested single-tube PCR assay (Chua et al. 1996; Evander et al. 1992). A non-nested PCR was also performed with the GP5+/GP6+ primers that amplify 150 bp products, a similar size as generated by the S14 PCR. Both PCR systems utilized 1.5 mM $MgCl_2$ and 0.4% BSA in a 50 µl buffer mix. The amplimers were run on 2% agarose gels and stained with 10 µg/ml EtBr at a concentration of 1 µl per 10 ml of agarose gel (Stock 10µg/ml).

The nested PCR was able to detect 1 copy of HPV 16 L1 plasmid when we mixed with DNA extracted from HPV negative smear (Chua et al. 1996). The limit of detection for SiHa DNA in each PCR run was consistently 1.0 fg or less.

For direct automated sequencing of the PCR products, the amplimers were purified with the QIA quick PCR Purification Kit (QIAGEN, Hilden, Germany). Another round of PCR utilizing the GP5+ and GP6+ fragment as template with 3.0 pmol of GP6+ primer was performed with the recommended amount of Big Dye Terminators (ABI Prism™ Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit, (PE

Biosystems, Foster city, Calif). Sequencing was performed using an ABI 310 sequencer (PE Biosystems).

The PCR analyses were performed blindly (coded). The samples were arranged in a manner ensuring that cases and controls were analyzed in the same analytic runs, to avoid the risk of assay performance variation (the most obvious risk being that of contamination) systematically biasing relationships of HLA or HPV status to case-control status.

Statistical methods

Odds ratio (OR) was calculated using the method of cross-products of 2X2 contingency tables. A two-sided *p*-value of <0.05 was considered to be significant. In case of small values, Fisher's exact test was performed to estimate the statistical significance using the approximation of Woolf. In paper II multivariable analyses were done using logistic regression (LogXout software) controlling for covariates.

RESULTS

Paper I: MICA allele 5 and 5.1 were the most common alleles in this population. No significant difference in MICA allele frequencies was found between patients and controls. When comparing patients (CIN I-III) by their HPV 16 and 18 statuses, an increased frequency of allele 5 was found in HPV 18 seropositive patients compared to HPV 18 seronegative patients (OR = 16.32; p = 0.01; CI, 1.78 –149.53). This difference was not significant when p-value was corrected for multiple comparisons. The risk tended to increase if the same individual also carries MICA A5 (10/74 patients, 13%, and 2/153, 1%, controls, OR = 9.68; 95% CI, 3.36 – 41.4; $p < 0.0005$) or A5.1 (12/74 patients, 16%, and 3/153 controls, 2%, OR = 11.8; 95% CI, 3.23 – 29.01; $p < 0.0005$).

Paper II: The *TNFa-11* allele was significantly more frequent in HPV16 seropositive patients compared with seronegative patients (OR, 5.40; *Pc* < 0.01; 95% CI, 1.9 – 15.3), but this significant finding was not observed in HPV18 seropositive patients. Similarly, the *TNFa-11* allele was also more frequent in DQ6-positive patients compared to DQ6-negative patients (OR, 9.31; *Pc* < 0.01; 95% CI, 2.99 –28.9). The *TNFa-2* allele was less frequent in DQ6-positive patients compared with DQ6-negative patients (OR, 0.20; *Pc* < 5, NS), but this was not significant after the *P* was corrected for multiple comparisons (13 multiple comparisons for *TNFa*). Multivariate analysis was also performed to confirm our observations. We calculated the frequency of HPV16 seropositives, DQ6, and *TNFa-11* carriers of genetic polymorphism in patients (64) and controls (147), and found that these markers together were significantly increased in the patients compared to the controls (OR, 15.05; *Pc* < 0.01; 95% CI, 4.49 –50.4).

Paper III: The two HLA haplotypes previously found to be associated with CIN, DQ6 and DR15, were also found to be clearly associated with invasive carcinoma of the cervix DR15 (OR = 3.73, CI, 1.87 - 7.42, $p < 0.0002$) and DQ6 (OR = 4.33, CI, 2.19 - 8.57, $p < 0.0001$). Both alleles in the DQ6 haplotype, DQA1*0102, and DQB1*0602 were associated with cervical cancer (OR = 4.92, CI, 2.64 - 9.18, $p < 0.0001$ and OR = 4.33, CI, 2.19 - 8.57, $p < 0.0001$ respectively.

The estimates of the population attributable proportion for cervical cancer for HLA-DQ6 and HLA-DR15 were 30.8% and 27.9%, respectively.

There was a tendency for over representation of HLA-DQ6 allele frequency among the patients with persistent HPV 16 positivity both for

the prediagnostic smear and biopsy compared to patients who were HLA-DQ6 negative (OR = 2.57, p = 0.18, CI, 0.81 - 8.15). Two alleles that were not part of the predefined study hypothesis, the DRB1 allele 07 and the DQB1 allele 0201, showed marginally significant negative associations with cervical cancer (OR = 0.46, CI, 0.21 – 0.98, p = 0.0476, p-value adjusted for multiple comparisons (pc) = 0.5712) and (OR = 0.53, CI, 028 – 1.01, p = 0.0628, Pc = 0.942) respectively.

Paper IV: None of the MICA or TNFa alleles were directly associated with cervical cancer. TNFa-11 frequency was higher among HPV 18 DNA positive patients compared to HPV 18 DNA negative patients (OR = 2.84, CI, 1.0391 - 7.7828, p = 0.0481). This association was not significant after the correction of the p-value for the number of the TNFa alleles compared. The HLA DQ6-TNFa-11 extended haplotype increases the risk for cervical cancer by three times (OR = 3.08, CI, 1.30 - 7.31, p < 0.0104). Infection with HPV16 in the patients with HLA DQ6-TNFa-11 haplotype is increasing the risk for the cancer of cervix by 23 fold (OR = 23.03, CI, 1.30 - 409.16, p < 0.0018). There was a minor over-representation of the TNFa-11 allele among the HPV16 DNA positive patients compare to controls (OR = 57.22, CI, 3.38 - 969,20, p < 0.0001).

DISCUSSION AND PERSPECTIVES

A large number of population-based studies have focused on the association of HLA genes with cervical cancer and CIN. However the interpretation of results is not straightforward, due to; **1)** HLA genes are polymorphic, and most studies are limited in size. As a result, the low number of observations for the individual alleles will limit the statistical power. **2)** Many studies have not been performed within an epidemiologically study design. For instance, controls and patients have not been matched for age and place of residence. In some cases, the controls have been drawn from different populations than the cases. **3)** A susceptibility factor may not be comparable in populations with different exposure. In the case of cervical neoplasia, it is assumed that certain HLA haplotypes may protect or increase the risk for disease when individuals are exposed to HPV. In any epidemiological study, the relation between exposure and susceptibility factor should be considered since any misclassification of the case and control groups will result in a weakening of the result between exposure and outcome.

Both in the CIN study (paper I and II) and in the CXCA study (paper III and IV), we studied the HLA genes in consideration of exposure to oncogenic HPV types to minimize the risk for selection biases**.** The only information loss encountered was due to the samples that DNA was not adequate for HLA or HPV typing.

Host genetic factors such as genes in the HLA region, could interfere with HPV infection at any stage of cervical tumorogenesis (Figure 6). This interference could happen at any stage of development of cervical tumors. We understand that women with persistent HPV infection are predisposed to develop cervical cancer. We also realize that women with high HPV 16 copy number (viral load) are at risk to develop cervical cancer (Josefsson et al. 2000; Ylitalo et al. 2000). The influence of HLA and immune system in the clearance of HPV infection would be at its peak when the copy number of virus is increased because more HPV antigen would be available for the HLA molecules to trigger the immune response. Since increasing viral load correlates with progression of cervical tumors and occurs in a relatively late stage, up-regulation of HLA expression would be very critical at this time. We assume that at the time of HPV infection and during the subclinical HPV latency, triggering of the immune responses is more dependent on the nonspecific part of immune reaction, such as cytokine production (TNF) or induction of stress induced cell surface molecules (MIC and HSP). In another words, it is presumed that when the HPV copy number is low, innate immunity

is critical. The classical HLA molecules would only be challenged when adequate antigen is available.

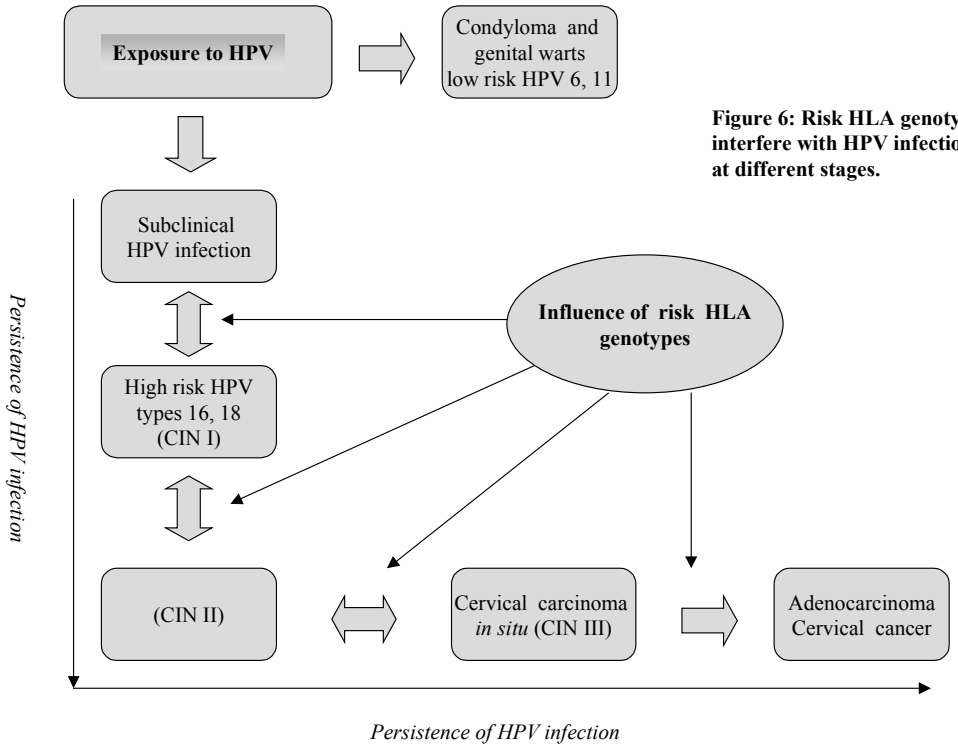

Figure 6: Risk HLA genotypes interfere with HPV infection at different stages.

This work suggests that the strongest association of HLA with cervical tumors is in the HLA class II region as both HLA-DQ6 and HLA-DR15 were found in a significantly higher frequency among the patients compared to the controls both in the CIN and in the CXCA study. MICA gene polymorphism, which is also associated with classical HLA I genes, did not show any association with either CIN or CXCA. We also conclude that TNFA gene polymorphism is correlated with HPV 16 seropositivity. It is difficult to say if this association is due to the level of TNFα production because in our CXCA study TNFA was not correlated with either HPV infection or the disease. A role of MICA in the pathogenesis of cervical tumors cannot be excluded despite the fact that polymorphism of this gene was not associated with cervical tumors. It would be interesting to analyze MICA expression in a post-transcriptional level in the cervical epithelium to see if it can contribute to regression of transformed lesions.

The association of specific HLA alleles and haplotypes with susceptibility to cervical tumors is probably attributable to a direct involvement of the HLA molecules as an antigen presenter, or possibly due to a close gene in linkage. Whatsoever knowledge about HLA genes as markers will provide a useful tool to identify the at-risk individuals.

Once the risk groups are identified, therapeutic and preventive peptide-based vaccines can be designed according to the specificity of any HLA molecule. For instance in the case of individuals carrying the risk HLA-DQ6 haplotype, specific HPV-derived peptides with good binding affinity to the HLA could be designed to stimulate immune responses against tumors.

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