Prognostic markers in head and neck carcinoma

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To my Grandpa Aaro

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. Well-known risk factors include tobacco smoking and alcohol consumption. Overall survival has improved, but is still low especially in developing countries. One reason for this is the often advanced stage of the disease at the time of diagnosis, but also lack of reliable prognostic tools to enable individualized patient treatment to improve outcome. To date, the TNM classification still serves as the best disease evaluation criterion, although it does not take into account the molecular basis of the tumor. The need for surrogate molecular markers for more accurate disease prediction has increased research interests in this field.

We investigated the prevalence, physical status, and viral load of human papillomavirus (HPV) in HNSCC to determine the impact of HPV on head and neck carcinogenesis. The prevalence and genotyping of HPV were assessed with an SPF10 PCR microtiter plate-based hybridization assay (DEIA), followed by a line probe-based genotyping assay. More than half of the patients had HPV DNA in their tumor specimens. Oncogenic HPV-16 was the most common type, and coinfections with other oncogenic and benign associated types also existed. HPV-16 viral load was unevenly distributed among different tumor sites; the tonsils harbored significantly greater amounts of virus than other sites. Episomal location of HPV-16 was associated with large tumors, and both integrated and mixed forms of viral DNA were detected. In this series, we could not show that the presence of HPV DNA correlated with survival.

In addition, we investigated the prevalence and genotype of HPV in laryngeal carcinoma patients in a prospective Nordic multicenter study based on fresh-frozen laryngeal tumor samples to determine whether the tumors were HPV-associated. These patients were also examined and interviewed at diagnosis for known risk factors, such as tobacco smoking and alcohol consumption, and for several other habituations to elucidate their effects on patient survival. HPV analysis was performed with the same protocols as in the first study. Only 4% of the specimens harbored HPV DNA. Heavy drinking was associated with poor survival. Heavy drinking patients were also younger than nonheavy drinkers and had a more advanced stage of disease at diagnosis.

Heavy drinkers had worse oral hygiene than nonheavy drinkers; however, poor oral hygiene did not have prognostic significance. History of chronic laryngitis, gastroesophageal reflux disease, and orogenital sex contacts were rare in this series.

To clarify why vocal cord carcinomas seldom metastasize, we determined tumor lymph vessel (LVD) and blood vessel (BVD) densities in HNSCC patients. We used a novel lymphatic vessel endothelial marker (LYVE-1 antibody) to locate the lymphatic vessels in HNSCC samples and CD31 to detect the blood microvessels. We found carcinomas of the vocal cords to harbor less lymphatic and blood microvessels than carcinomas arising from sites other than vocal cords. The lymphatic and blood microvessel densities did not correlate with tumor size. High BVD was strongly correlated with high LVD. Neither BVD nor LVD showed any association with survival in our series.

The immune system plays an important role in tumorigenesis, as neoplastic cells have to escape the cytotoxic lymphocytes in order to survive. Several candidate HLA class II alleles have been reported to be prognostic in cervical carcinomas, an epithelial malignancy resembling HNSCC. These alleles may have an impact on head and neck carcinomas as well. We determined HLA-DRB1* and -DQB1* alleles in HNSCC patients. Healthy organ donors served as controls. The Inno-LiPA reverse dot-blot kit was used to identify alleles in patient samples. No single haplotype was found to be predictive of either the risk for head and neck cancer, or the clinical course of the disease. However, alleles observed to be prognostic in cervical carcinomas showed a similar tendency in our series. DRB1*03 was associated with node-negative disease at diagnosis. DRB1*08 and DRB1*13 were associated with early-stage disease; DRB1*04 had a lower risk for tumor relapse; and DQB1*03 and DQB1*0502 were more frequent in controls than in patients. However, these associations reached only borderline significance in our HNSCC patients.

List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I. Koskinen WJ*, Chen RW*, Leivo I, Mäkitie A, Bäck L, Kontio R, Suuronen R, Lindqvist C, Auvinen E, Molijn A, Quint WG, Vaheri A, Aaltonen L-M. Prevalence and physical status of human papillomavirus in squamous cell carcinomas of the head and neck. *Int J Cancer 2003;107:401-406* (*Equal contribution of authors)

II. Koskinen WJ, Brøndbo K, Mellin Dahlstrand H, Luostarinen T, Hakulinen T, Leivo I, Molijn A, Quint WG, Røysland T, Munck-Wikland E, Mäkitie AA, Pyykkö I, Dillner J, Vaheri A, Aaltonen L-M. Alcohol, smoking and human papillomavirus in laryngeal carcinoma: a Nordic prospective multicenter study. *Submitted*

III. Koskinen WJ, Bono P, Leivo I, Vaheri A, Aaltonen L-M, Joensuu H. Lymphatic vessel density in vocal cord carcinomas assessed with LYVE-1 receptor expression. *Radiother Oncol 2005;77:172-175*

IV. Koskinen WJ, Partanen J, Vaheri A, Aaltonen L-M. HLA-DRB1, -DQB1 alleles in head and neck carcinoma patients. *Tissue Antigens 2006;67:237-240*

Abbreviations

BVD	blood vessel density
CDK	cyclin-dependent kinase
CR	cumulative relative survival
CI	confidence interval
DEIA	DNA enzyme immunoassay
E	early gene
EGF	epidermal growth factor
FGF	fibroblast growth factor
GERD	gastro-esophageal reflux disease
HLA	human leukocyte antigen
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HR	hazard ratio
L	late gene
LiPA	line probe assay
LSCC	laryngeal squamous cell carcinoma
LVD	lymphatic vessel density
LYVE-1	lymphatic vessel endothelial marker 1
MHC	major histocompatibility complex
OS	overall survival
PCR	polymerase chain reaction
PFS	progression free survival
PlGF	placental growth factor
pRb	retinoblastoma protein
RRP	recurrent respiratory papillomatosis
SPF	short PCR fragment
TNM	T - extent of primary tumor N - absence or presence and extent of regional lymph node metastasis M - absence or presence of distant metastasis
UICC	International Union Against Cancer
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

Introduction

Head and neck squamous cell carcinomas (HNSCCs) are known to have a multifactorial etiology. Well-established risk factors include tobacco and alcohol use and in some areas betel quid chewing as a risk for oral carcinoma. Other risk factors proposed include poor nutritional status, genetic susceptibility, and, in a subset of HNSCCs human papillomavirus (HPV). Overall survival of patients who have contracted the disease has improved over the last two decades, but diagnostic tools capable of predicting tumor growth are needed for individualized treatment. To date, the best prognostic marker in use is the TNM classification (UICC), which is based mainly on tumor size. This classification is not able to distinguish neoplasias with infiltrative growth potential from those with restricted local growth. The combined risk effect of alcohol and tobacco is not only additive, but it seems to be multiplicative.[1,2] Heavy alcohol users may also have worse survival than nonheavy users, but it is difficult to separately analyze the molecular changes leading to this; alcohol may act as a solvent for other carcinogens, and the metabolite acetaldehyde has been shown to have carcinogenic properties.[3,4]

HPVs are small double-stranded DNA viruses featuring oncogenic properties. They infect mucosal and skin epithelia. In cervical carcinomas, HPV is needed for malignant transformation.[5,6] Knowledge about HPV infections in HNSCC has increased in the last twenty years. Studies suggest that the subset of HNSCCs positive for HPV DNA has a better survival than that which is HPV-negative.[7,8] HPV-positive HNSCC is hypothesized to be different tumor entity from HPV-negative HNSCC.[9] While HPV may alter tumor behavior, its prognostic value in HNSCC remains ambiguous.[10,11]

Neoplastic cells need angiogenesis to grow beyond one cubic millimeter.[12] Induced angiogenesis also requires lymphatic drainage. Solid tumors tend to metastasize via lymphatics. Tumors originating from the upper aerodigestive tract tend to metastasize to lymph nodes residing in the neck area.[13] This has a drastic effect on survival.[14] Early-stage disease (T1-T2) at diagnosis represents a curable disease managed with one treatment modality, but when neck lymph nodes become involved (N1-N2) the treatment is

combined modality and the probability of a tumor-free outcome decreases. Tumors originating from different subsites in the head and neck have different metastatic potential; one explanation for this may be differences in blood and lymphatic microvasculature.

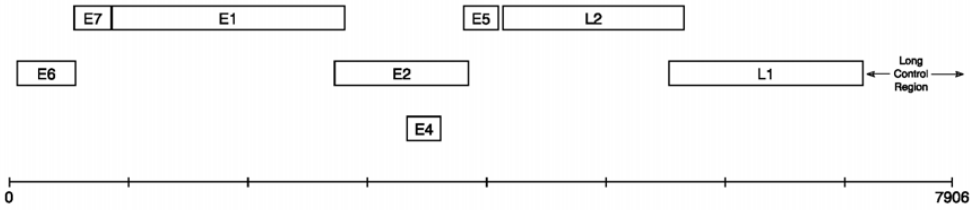
Human leukocyte antigens (HLAs) play a role in cervical carcinoma, a mucosal HPV-related cancer. Several HLA-class II haplotypes have been found to be predictors of decreased[15-18] or increased[19,20] risk for cervical cancer. The well-known risk haplotype is DQB1*0301-03,[16,21,22] whereas DRB1*13 and DQB1*0603[23-25] seem to be protective haplotypes. HNSCC share features with cervical carcinomas, and the same HLA class II alleles may play a role in both malignancies. Certain alleles have been found to be prognostic in recurrent respiratory papillomatosis (RRP),[26-28] disease associated with HPV 6 and HPV11.

Review of the literature

1. Human papillomavirus (HPV)

HPVs are small, nonenveloped, double-stranded DNA viruses. They belong to the *Papillomaviridae* family. Their genome is circular and about 7.9 kb in size, comprising six early (E) genes and two late (L) genes. To date, over 100 papillomavirus types have been sequenced.[29] Papillomaviruses are found in many species, including birds and cattle, but humans are the most studied host.

Figure 1.



A schematic presentation of HPV-16 genome indicating the organization of early (E) and late (L) genes.

1.1. Cutaneous and mucosal HPVs

Cutaneous HPV types (2, 4, 7) infect skin epithelia, causing warts in different parts of skin depending on the HPV type.[30] Epidermodysplasia verruciformis is a rare hereditary disease in which infections with HPV-5 and -8 cause skin carcinoma.[31] The role of HPV in skin cancers of immunocompetent patients is not fully understood.[32,33]

Mucosal HPV types (6, 11, 16, 18) infect the anogenital area and the upper respiratory tract, causing condylomas and anogenital warts,[34,35] papillomas in the oral cavity, nose, and nasopharynx, inverted papillomas of the nose and paranasal sinuses,[36] and laryngeal papillomas.[37,38] They are also related to certain malignant tumors in these anatomical areas.

1.2. High- and low-risk HPVs

HPV classification into high- and low-risk types is based on molecular epidemiological studies and functional evidence of the oncogenic potential of certain HPV types.[39] The high-risk HPV types (16, 18, 31, 33, 35, 52, 58, 59, 68, 73, and 82) are frequently associated with invasive squamous cell carcinoma; the low-risk types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81) are rarely found in carcinomas and are often associated with premalignant or benign disease.

1.3. HPV life cycle and cancer

HPV infects proliferative basal epithelial cells and has access to them usually through microlesions. The E5, E6, and E7 have a proliferation-stimulating effect, and they act in the early phase of infection. At suprabasal layers, the L1 and L2 genes start to act in viral assembly and release of virions with the shearing epithelial cells. In productive infection, the viral genome stays episomal.

In carcinomas, part of the HPV genome integrates into the host cell genome.[40] During the integration part of the E2 sequence and the E4 and E5 coding sequences are deleted. The E5 gene is expressed only before integration, and its functions are important in the early phase of infection. However, in experimental models, it has been shown to promote transformation-associated changes, making cells less dependent on epidermal growth factor (EGF).[41-43] The oncogenic E6 gene affects functions of several cellular proteins by binding them,[44,45] and it is also capable of immortalization of distinct cell lines on its own.[46] The first oncogenic effect reported for E6 was p53 binding and subsequent inhibition of apoptotic signals,[47,48] and another important function is telomerase activation.[49] E7 is the main transforming gene capable of inactivating the retinoblastoma gene product,[50,51] which releases the cell from growth arrest to the S phase.[52] It also suppresses CDK inhibitors[53] and activates cyclins E and

A.[54] After viral integration into the host genome, E2 gene repression[55,56] of the E6 and E7 promoters ceases and synergistic function in transformation begins. These oncogenic changes are properties of high-risk HPV E6 and E7 genes; the counterparts in low-risk HPV are unable to immortalize cells.[57,58] However, E6 and E7 of high-risk HPVs are not capable of converting normal cells to a malignant phenotype without chemical and physical changes.[59]

The first associations between HPV and malignant tumors were reported in 1974, when zur Hausen discovered HPV in cervical carcinoma.[34] Later convincing epidemiological evidence has linked HPV to cervical carcinogenesis.[5,60-63] The first cases of HPV in upper aerodigestive tract cancers were reported at the beginning of the 1980s by Syrjänen et al.,[64,65] who proposed that HPV was involved in oral squamous cell cancers.

2. Angiogenesis in cancer

2.1. Angiogenic properties of tumorigenesis

Growth of normal and neoplastic cells is dependent on angiogenesis.[12,66,67] Ability to induce blood vessel growth is not intrinsic to proliferating cells. To progress to a size over one cubic millimeter, neoplasias must gain angiogenic properties.[68-71] In normal adult life, angiogenesis takes place only in pathological events, with one exception, the highly controlled female reproductive cycle.[72]

At the *in situ* level, the neoplastic cells are influenced by angiogenic molecules; vascular endothelial growth factor and acidic and basic fibroblastic growth factors (VEGF, aFGF, bFGF), and anti-angiogenic factors (endostatin, thrombospondin-1, angiostatin), and when the balance between these factors is maintained, angiogenesis does not exist. As the neoplastic cells change to a more invasive phenotype and angiogenic signals prevail over anti-angiogenic signals ("angiogenic switch"), the tumor becomes vascularized and is able to maintain sustained angiogenesis, one of the hallmarks of cancer.[71,73] This presumable "halfway" event in carcinogenesis has led researchers to study tumor microvessel densities with endothelium-specific antibodies (CD31, von Willebrand factor). Blood vessel density (BVD) is a prognostic factor in breast, prostate, and head and neck carcinomas.[74-76] These findings have launched

studies of angiogenesis inhibitors; if vascular growth could be prevented, the neoplastic mass would merely be a manageable chronic disease.[77]

Anti-angiogenic treatments include antibodies, soluble receptors, small-molecule tyrosine kinase inhibitors, antisense oligonucleotides, aptamers, and RNA interference. The most studied molecule in anti-angiogenic treatment is monoclonal antibody against VEGF, bevacizumab (Avastin®; Genentech Inc.). In phase III trials, bevacizumab has been shown to increase overall survival (OS) and/or progression-free survival (PFS) in colorectal, breast, and lung cancer when combined with cytotoxic agents.[78] However, the function of direct and indirect anti-angiogenic therapy is still poorly understood, and in clinical trials, these drugs behave unpredictably from phase I results. Jain et al.[79] have summarized the recent results of phase III trials of anti-angiogenic drugs. They conclude that anti-angiogenic therapy may be effective in treating solid tumors, but the treatment must be well planned for a specific patient group, which is a challenge for clinicians. In addition, no useful marker exists to evaluate the efficacy of anti-angiogenic therapy during the treatment period.

Table 1. Vascular endothelial growth factors and their receptors.

VEGF receptors	VEGFR-1 (Flt1)	VEGFR-2 (Flk1/KDR)	VEGFR-3 (Flt4)	Neuropilin-1
VEGF family members	VEGF VEGF-B PlGF	VEGF VEGF-C VEGF-D	VEGF-C VEGF-D	VEGF

2.2. Lymphangiogenic properties of tumorigenesis

Tumor angiogenesis is crucial for tumor growth, and it potentially promotes hematogenous metastasis;[80] however, lymph node metastasis is clinically the most important prognostic sign.[81] Solid tumors tend to disseminate to sentinel lymph nodes via lymphatic drainage. Tumor lymphangiogenesis and its relation to lymphatic metastasis are not fully understood.[82,83] Studies show that

a family of vascular endothelial growth factors (VEGF-(A), B, C, D and placental growth factor) and their receptors (VEGFR-1, 2, 3, and neuropilin-1) play a major role in angio- and lymphangiogenesis.[84] Of these, VEGF-C and -D promote lymphangiogenesis through activation of VEGFR-3. [85-87]

In experimental studies, VEGF-C and -D are reported to speed up tumor growth and promote spreading of tumor cells via the lymphatics.[88,89] In clinical studies, VEGF-C and -D are described as prognostic at different stages of cervical carcinoma,[90] and VEGF-D is an independent prognostic marker for colorectal carcinoma[91] and epithelial ovarian carcinoma.[92] In these studies, high VEGF-D expression in tumor tissues predicted a poor prognosis; epithelial ovarian carcinoma patients with a high VEGF-D-expressing tumor a had poor carcinoma-specific survival (RR 8.2 95% Cl 2.33-83.33).

These results reveal that the lymphatic drainage system is an accessible route of local metastasis. Based on recent data, peritumoral lymphatics appear to be more important in tumor dissemination than intratumoral lymph vessels.[93,94]

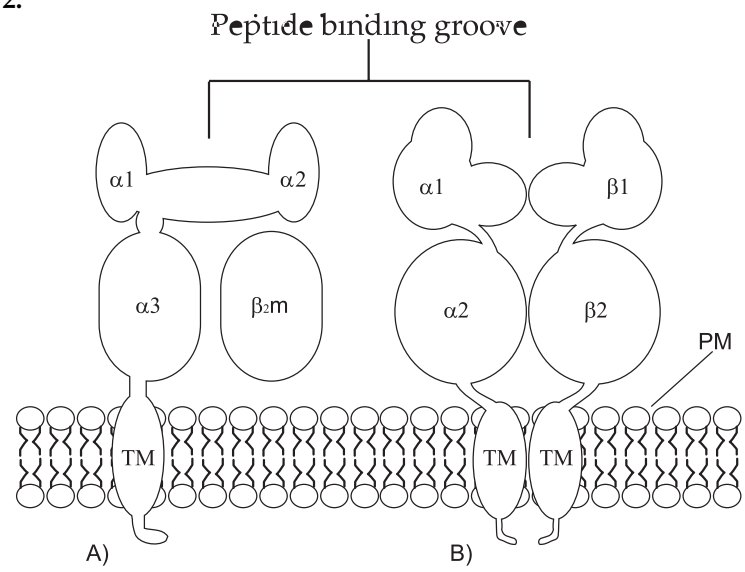
3. The HLA system

3.1. HLA classification

The human leukocyte antigen (HLA) complex, located in chromosome 6, comprises 200 genes, over 40 of which encode leukocyte antigens.[95] Other genes in the loci are not related to HLA; however, some are involved functionally with the immune system.

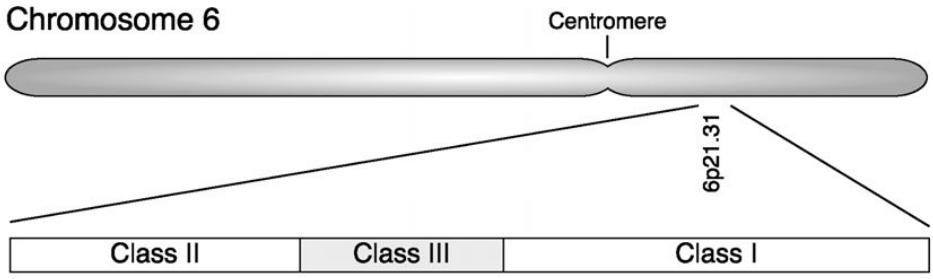
The HLA genes functioning in immune response are categorized as either class I or class II; these classes differ in structure and function.

Figure 2.



A simplified illustration of the structure of HLA class I (A) and class II (B) molecules. β_2-microglobulin (β_2m) is the light chain of the class I molecule. The α-chain of the class I molecule has two peptide binding domains (α1 and α2), an immunoglobulin-like domain (α3), a transmembrane region (TM), and a cytoplasmic tail. Each of the class II α- and β-chains has four domains: the peptide-binding domain (α1 or β1), the immunoglobulin-like domain (α2 or β2), the transmembrane region, and the cytoplasmic tail. PM=plasma membrane (modified from Klein J & Sato A, NEJM 2000;343:702-9).

Figure 3.



An overview of the HLA locus on chromosome 6 (modified from Klein J & Sato A, NEJM 2000;343:702-9).

There are approximately 20 class I genes in the HLA loci, and three of these, HLA-A, -B, and -C, termed classic genes, are the key players in immunology. The loci of class II genes on chromosome 6 are determined with three letters: the first (D) indicates the class, the second (M, O, P, Q, or R) is the family, and the third (A or B) is the chain (α or β). For example, HLA-DQB are class II genes of the Q family coding for β-chains. The individual genes are differentiated by Arabic numbers, and for separate numerous allelic variants of these genes is a number preceded by an asterisk. HLA-DQB1*0303 stands for allelic variant 0303 of gene 1, which encodes the β-chain of a class II molecule belonging to the Q family. The function of both class I and class II molecules is the presentation of pathogen-derived peptides to T-cells, thereby initiating the adaptive immune response.

3.2. HLA and disease implications

Several human diseases are known to be associated with HLA. Distinct patterns of HLA alleles display predispositions to certain diseases or protection against them. Although associations observed in one population may not be found in other geographic regions or ethnic groups, worldwide screening reports are important in prediction of disease susceptibility and resistance.

In autoimmune disease, failure to discriminate between self- and nonself antigens leads to an immune response raised against autologous antigens of normal body tissues. An HLA association has been recognized in type 1 diabetes,[96] ankylosing spondylolitis,[97] and celiac disease.[98] Selected associations between HLA markers and autoimmune diseases are listed in Table 2.

Table 2. HLA associations in selected autoimmune diseases (modified from Klein J & Sato A, NEJM 2000;343:702-9).

HLA marker	Associated disease	Relative risk
B27	Ankylosing spondylolitis	87.4
B27	Reactive arthropathy, including Reiter's syndrome	37
DR3	Insulin-dependent diabetes mellitus	3.3
DQB1*0201		2.4
DR4		6.4
DQB1*0302		9.5
DR2		0.19
DQB*0602		0.15
DR3	Celiac disease	10.8
DR7,11		6-10
DR3	Dermatitis herpetiformis	15.9
DR3	Systemic lupus erythematosus	5.8

Studies show associations between infectious diseases and HLA genes as well. Specific class I and II alleles are able to protect against a severe form of malaria,[99] clearance from hepatitis B[100,101] and C[102,103] is associated with distinct HLA alleles. Certain HLA alleles are also associated with susceptibility to persistence of these infections.[102,104] An association with severe hantavirus infections in subjects carrying a certain HLA haplotype has been reported.[105]

HLA alleles predisposing to[23] and protecting from[106] cervical HPV infections exist.

Generally, HLA class I disease associations involve cytotoxic T-lymphocytes, whereas class II disease associations involve T-helper or -suppressor lymphocytes. The reported disease associations seem to share an interesting feature: associations that have been reproduced in different studies are mainly linked to autoimmune diseases, while those connected to infectious diseases apparently cannot be easily reproduced.[107] This may be due to differences in determining diseases and different patient selection criteria.

3.3. HLA and cancer

The involvement of the HLA system in cancer is not completely understood. HLA and cancer prognosis used as search terms produces thousands of articles about blood borne malignancies and hematopoietic diseases, but the immune system plays an important role in development of solid tumors as well.

Carcinoma cells tend to express several surface proteins different from those of their normal counterparts. Despite their abnormal behavior, they are inefficiently recognized by cytotoxic T-cells, a possible result of cancer cells downregulating MHC translation, but one may wonder why these abnormal cells are not terminated by natural killer cells.[108] Escape from the immune system requires a complex modulatory capability, and several carcinomas have been shown to possess these features. Downregulation or alterations in HLA class I expression are reported in breast cancer,[109] lung cancer,[110] cancer of the uterine cervix,[111] and HNSCC.[112] This is thought to be one of the escape mechanisms from immune attack.[113] Studies show impaired antigen processing in HNSCC,[114,115] and in addition dendritic cell maturation and a T-cell subset imbalance[116] lower the ability of the immune system to eliminate neoplastic cells.

HLA class II associations have been investigated widely in cervical carcinomas, an epithelial malignancy resembling HNSCC. Studies have reported candidate alleles and haplotypes associated with risk for or protection from cervical carcinoma and with disease outcome. To date, the strongest protective association in these malignancies has been DRB1*13 and/or DQB1*0603 alleles. The haplotype possessing the highest risk for cervical cancer

is DRB1*1501-DQB1*0602. These findings were recently reviewed by Hildesheim.[17] Comprehensive population-based studies have revealed discrepant results considering risk alleles in cervical cancers,[15,16,23] with candidate alleles varying between geographical areas, disturbing worldwide risk mapping.

However, cervical carcinogenesis is also largely influenced by persistent HPV infection, thus when estimating the impact of distinct HLA alleles on cervical cancer risk, HPV status should be considered. HPV has been reported to decrease the number of dendritic Langerhans cells in the female genital tract.[117] Moreover, HPV-positive cervical carcinomas are influenced by different HLA alleles than their HPV-negative counterparts. Studies show over-representation of certain class II alleles in patients with HPV-positive cervical carcinomas[25] and cervical intraepithelial lesions,[22,25,118] which implicates poor viral clearance in patients carrying these alleles. Few studies exist on the class II associations in HNSCC.

4. Head and neck cancer

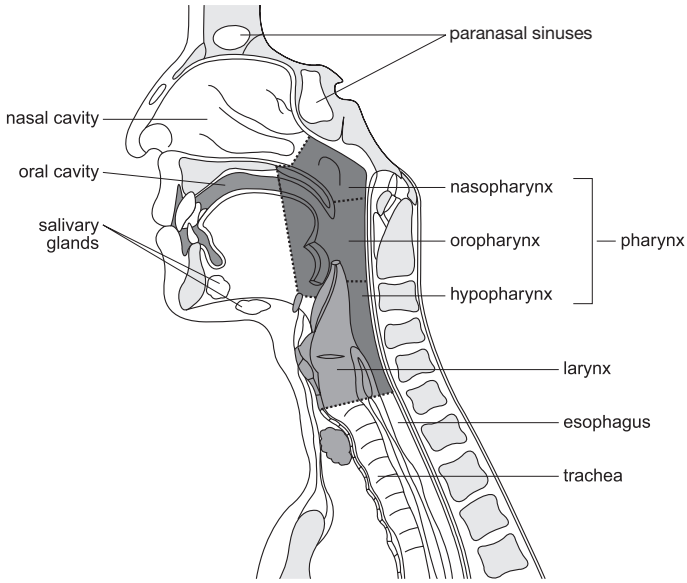
Head and neck squamous cell carcinoma (HNSCC) comprises epithelial malignancies arising from the mucosa of the lips, oral cavity, oropharynx, nasopharynx, hypopharynx, larynx, maxillary sinus, nasal cavity, ethmoid sinus, and salivary glands. It is the fifth most common cancer in males and the eight most common in females.[119] The treatment of HNSCC is related to tumor extent and function preservation; surgery or radiotherapy alone or in combination with is the traditional treatment option, but use of concomitant chemoirradiation is increasing due to significantly improved locoregional control and overall survival.[120-125]

4.1. Etiology and risk factors

The annual total number of new HNSCC cases globally is over 600 000, with 350 000 deaths occurring.[119] The total number of new cases in Finland in 2003 was over 600, and the annual death rate about 200.[126] For most head and neck cancer sites, the age-adjusted incidence rate is higher in men than in women;

the estimated worldwide number of new cases of head and neck cancers in 2002 was 477 000 in males and 166 000 in females according to global cancer statistics.[119] The worldwide number of new laryngeal cancer in 2002 was 160 000; in Finland the corresponding figure in 2002 was 110 and this cancer was much more common in males. The age-adjusted incidence rate in males has decreased from 6.2 to 2.2/100 000 individuals between the 1960s and present; the age- adjusted incidence rate in females for the same period has changed little, from 0.4 to 0.2/100 000 individuals.[126]

Figure 4.



A sagittal view of the head and neck region (modified from Syöpätaudit 3rd. Ed., Duodecim).

The etiology of HNSCC is considered multifactorial. Tobacco and alcohol abuse are the best-known risk factors.[127-129] Results of selected case-control studies[1,127,130] on the risk of tobacco smoking and alcohol are presented in Table 3. Ethanol itself is not carcinogenic,[131] but acetaldehyde, a major metabolite of ethanol, is carcinogenic in animals.[4,132] Studies show that acetaldehyde may be a

significant carcinogen also in humans.[133,134] Current data reveal that acetaldehyde levels in saliva are higher in tobacco smokers than in nonsmokers and that smoking combined with alcohol consumption may increase salivary acetaldehyde levels up to 7-fold.[135] This increases the the carcinogenic exposure of the epithelium.

Table 3. Results from selected case-control studies evaluating the risk OR (95% CI) of tobacco smoking and alcohol consumption in head and neck cancer.

	Tumor site					
	Oropharynx	Oral cavity	Pharynx	Larynx	Dose	Ref.
Heavy smoking		14.3[a] (4.2-48.0)	17.6 (4.1-74.7)	7.1 (3.3-15.4)	≥ 25 cigarettes/day	127
	2.8[b] (1.8-4.4)				≥ 40 cigarettes/day	130
				42.9 (22.8-80.9)	≥ 25 cigarettes/day	1
Heavy drinking		3.4 (1.7-7.1)	3.6 (1.8-7.2)	2.1 (1.2-3.8)	≥ 60 drinks/wk	127
	8.8[b] (5.4-14.3)				≥ 30 drinks/wk	130
				5.9 (3.1-11.3)	≥ 56 drinks/wk	1
Combined		79.6[c] (NA)		11.7 (NA)		127
	37.7[b] (NA)					130
				177.2		1

[a] Results for male only; [b] Entire study population was male; [c] Oral cavity and pharynx combined; NA - data not available

Evaluating the independent risk of alcohol is difficult because many heavy alcohol users are also smokers. Studies have shown tobacco smoke to pose a greater risk than alcohol for laryngeal squamous cell carcinoma (LSCC); while alcohol intake without tobacco smoke possesses a risk for LSCC, it is far lower than that of tobacco alone.[136]

Environmental tobacco smoke has also been reported to increase the risk for HNSCC.[137,138] In addition betel quid (Areca nut) chewing is a major risk

factor in some areas of Asia,[139-141] and studies show that, besides oral carcinoma, it is also an independent risk factor for carcinomas of the upper aerodigestive tract.[142] A history of gastroesophageal reflux disease (GERD) has been reported to increase the susceptibility to a subset of HNSCC.[143] Dietary risk for HNSCC is high in people consuming low levels of fruits and vegetables.[144-146] High intake of fiber and vitamin C has a positive effect on survival from laryngeal and hypopharyngeal cancers.[147] However, assessing the influence of nutrition is difficult because a large proportion of HNSCC patients are malnourished,[148,149] and it is not known which micronutrients actually prevent the carcinogenesis. Genetic susceptibility has also been proposed as an etiological factor.[150,151] Studies have shown increased cancer risk in HNSCC patients' relatives. Inherited malfunction of the immune system, DNA repair systems, and cell cycle control mechanisms may increase the susceptibility to malignant growth. Occupational risk factors include hardwood dust, furniture making, and leather tanning, which mainly cause adenocarcinomas of the sinonasal area, but to some extent also pose a risk for squamous cell carcinomas.[152-154]

4.2. Tumor classification

Head and neck tumors are categorized according to the TNM classification (Tables 1 and 2), which is based on guidelines set by the International Union Against Cancer (UICC). The classification varies slightly depending on the subsite of tumor origin. Generally, T is a measure of primary tumor mass, N indicates whether lymph nodes are involved, and M indicates for distant metastasis.

Table 4. Tumor classification for glottic laryngeal cancer (UICC 2002).

TNM	Tumor characteristics
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour limited to vocal cord(s)(may involve anterior or posterior commissure) with normal mobility
a	Tumour limited to one vocal cord
b	Tumour involves both vocal cords
T2	Tumour extends to supraglottis and/or subglottis, and/or with impaired cord mobility
T3	Tumour limited to larynx with vocal cord fixation and/or invades paraglottic space, and/or with minor thyroid cartilage erosion (e.g. inner cortex)
T4	
a	Tumour invades through the thyroid cartilage, or invades tissues beyond the larynx, e.g., trachea, soft tissues of the neck including deep/ extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), strap muscles, thyroid, oesophagus
b	Tumour invades prevertebral space, mediastinal structures, or encases carotid artery
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in single ipsilateral lymph node, 3cm or less in greatest dimension
N2	
a	Metastasis in a single ipsilateral lymph node, more than 3cm but not more than 6cm in greatest dimension
b	Metastasis in multiple ipsilateral lymph nodes, none more than 6cm in greatest dimension
c	Metastasis in bilateral or contralateral lymph nodes, none more than 6cm in greatest dimension
N3	Metastasis in a lymph node more than 6cm in greatest dimension
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 5. Stage classification for glottic laryngeal carcinoma (UICC 2002).

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T1-2	N1	M0
	T3	N0-1	M0
Stage IVA	T1-3	N2	M0
	T4a	N0-2	M0
Stage IVB	T4b	Any N	M0
	Any T	N3	M0
Stage IVC	Any T	Any N	M1

4.3. HPV in head and neck squamous cell carcinoma (HNSCC)

Morphological findings suggested involvement of HPV in oral squamous cell carcinomas at the beginning of the 1980s.[65] Some years later, HPV16 DNA was identified with a hybridization technique in oral carcinoma.[155] After these initial findings, knowledge about viral etiology in HNSCCs has increased dramatically.[156-158] The most frequently detected high-risk HPV is type 16.[159] HPV-18 and 33 are also found in head and neck malignancies, but their incidence is lower than that of HPV-16. HPV infection is an important and obligatory step in cervical carcinogenesis. When information about HPVs ability to promote malignant transformation at the cellular level emerged, it was considered to play a key role in a subset of head and neck cancers as well.[157] The prevalence of HPV in HNSCC varies depending on the detection method used and on the tumor subsite. However, several studies have discovered high prevalences in oropharyngeal tumors, especially tonsillar carcinomas,[160-163] whereas in laryngeal carcinomas great variation exists.[157,164-167] Nevertheless, HPV DNA is less frequently present in laryngeal than in oropharyngeal malignancies. Studies show relatively low prevalence of HPV infection (16%) also in hypopharyngeal carcinomas.[11,157,168] In contrast to cervical carcinomas, the viral copy numbers in HNSCC, except in tonsillar carcinomas, are low.[169-171] Some studies have found HPV to have a positive influence on prognosis,[157,172] but contradictory reports also exist.[173,174]

HPV seroprevalence has been shown to be prognostic in head and neck cancer.[175] The HPV-16 seropositivity in this study was twice as high in patients than in controls. The odds ratios for head and neck cancer in HPV-16 seropositive patients varied depending on the subsite, the highest being oropharyngeal cancer and cancer of the base of the tongue.

At the molecular level, HPV-positive HNSCC displays different characteristics than HPV-negative HNSCC. HPV-positive tumors have wild-type p53,[176] decreased expression of cyclin D and pRb, and upregulation of p16,[177-179] whereas HPV-negative tumors have not. These and other possible molecular differences alter the behavior of the tumor and affect the prognosis.

4.4. Lymphatic vessels and tumor metastasis in HNSCC

HNSCC are known to metastasize via the lymphatics.[13] Nodal involvement of a tumor is often associated with a more complex treatment. It would be useful if tumors with a disseminating potential could be characterized already at diagnosis.

Increased understanding of the importance of lymphatic spread to tumor growth has launched studies of this topic in HNSCC also. However, the results are somewhat ambiguous. Some studies show peritumoral lymphatic vessels as prognostic of lymph node involvement,[180,181] while others have found an association with better survival.[182] Most recently intratumoral lymphatics have been presented as predictors of poor outcome.[181-185] Interestingly, a recent review by Alitalo et al.[186] states that lymphatics lying in the tumor periphery are the most important in tumor dissemination. HNSCC is a heterogeneous group of tumors arising from the upper aerodigestive tract, and the density of the lymphatic vessel network may differ according to the tumor subsite.

4.5. HLA class II associations in head and neck tumors

Recent studies reveal associations of class II alleles in recurrent respiratory papillomatosis (RRP), a laryngeal premalignant condition caused by HPV-6 and -11. Individuals carrying DRB1*0102 have an increased risk for RRP,[27] and DQA*0102 and DQA*0501/DQB1*0201 are risk alleles in the white American population.[28] Aaltonen et al.[26] identified DQB1*0501 to be protective against adult-onset laryngeal papillomatosis in a distinct subgroup of patients. Recurrent respiratory papillomas seldom progress to invasive carcinomas[187,188] and are genetically separate from invasive carcinomas.[189] Potential risk alleles in carcinogenesis or alleles associated with disease outcome may differ from those described in RRP.

High-resolution HLA class II screenings in HNSCC are few. Tisch et al.[190] determined HLA-DR6 antigens from 141 HNSCC patients and found HLA-DR6 to be associated with poor 5-year survival. The 5-year survival rate for HLA-DR6-positive patients was 40%, whereas for HLA-DR6 negative patients it was

60%. Nevertheless, studies with adequate patient series and high-quality methods are required to assess risk alleles and haplotypes in HNSCC.

Aims of the study

Head and neck cancer is considered etiologically multifactorial. The most commonly used prognostic tool for clinicians is the TNM classification, which does not take into account the biological properties of the tumor. The surrogate molecular markers in HNSCC could enable more individualized treatment planning and effective therapy.

HPV is claimed to be involved in as many as 100 000 HNSCC cases worldwide per year, the majority of which are oropharyngeal, especially tonsillar cancers. When part of the HPV genome integrates into the host genome, the viral gene products are able to disrupt the cell cycle, leading to host cell changes towards a malignant phenotype. The major risk and prognostic factors for HNSCC are tobacco and alcohol use and it is not fully understood why HPV is less frequently present in other sites in the head and neck than the oropharynx. HPV-associated HNSCC has been suggested to be a different disease entity than that without viral DNA.

A major issue influencing prognosis in HNSCC is tumor dissemination; if a tumor with high-metastazing potential is detected early, treatment and follow-up can be organized efficiently.

The immune system is able to detect and eliminate abnormal cells and foreign objects. Neoplastic cells change their phenotype when developing the ability to escape apoptosis and cell senescence. If the immune system is unable to recognize these cells, tumor development is evident. HLA molecules play a key role in the recognition of structures by the immune system. The individual alleles or haplotypes have been demonstrated to have prognostic value in several malignancies, but their impact on head and neck carcinogenesis is unknown.

The specific objectives of this thesis were as follows:

I. To determine the prevalence and physical status (episomal or integrated viral DNA) of human papillomavirus in fresh-frozen specimens of primary HNSCC.
II. To evaluate the impact of risk factors on the clinical outcome of laryngeal carcinoma in a prospective Nordic multicenter study.
III. To analyze blood and lymphatic microvessel densities in primary HNSCC tumor specimens, with a special focus on rarely metastasizing vocal cord carcinomas.
IV. To investigate the effect of HLA class II allelic variance on head and neck carcinogenesis.

Patients and methods

1. Patients (I-IV)

Subjects in the studies were not consecutive. All patients fulfilling inclusion criteria in each study were recruited to avoid a selection bias. In Study I, HNSCC patients treated at Helsinki University Central Hospital during 1993-2002 whose fresh-frozen samples were available and submitted at diagnosis to ENT Clinic Tumor Bank participated. In Study II, LSCC patients who provided tumor samples for HPV analysis at diagnosis during 2000-2003 were recruited. They were from Helsinki University Central Hospital, Rikshospitalet University Hospital, Oslo, and Karolinska University Hospital, Stockholm. In Study III, samples were obtained from HNSCC patients who were diagnosed and treated at Helsinki University Central Hospital during 1998-2001 and whose samples were submitted to theENT Clinic Tumor Bank. In Study IV, the blood samples collected for the ENT Clinic Tumor Bank from HNSCC patients diagnosed and treated during 1997-2004 at Helsinki University Central Hospital were utilized. All study protocols were approved by the Ethics Committee of each hospital. Patient characteristics are presented in Table 6

2. Methods

2.1. DNA extraction (I, II, IV)

A fresh-frozen tissue sample was obtained during the tumor operation (Studies I and II), and the tumor specimen was confirmed by a pathologist to contain a minimum of 40% (Study I) or 20% (Study II) neoplastic cells.

DNA extraction was performed using a Qiagen DNA mini kit (Qiagen®, GmbH, Hilden, Germany) and tested for proper DNA quantity and quality with routine β-globin PCR.

The buffy coat fraction was separated from each patient's peripheral blood sample and used for DNA extraction (Study IV) with a FlexiGene DNA extraction kit (Qiagen®, GmbH).

Table 6. Patient characteristics in Studies I-IV.

Characteristics			Study I	Study II	Study III	Study IV
					n (%)	
Origin						
	Finnish		61 (100)	11 (16)	60 (100)	162 (100)
	Swedish			6 (9)		
	Norwegian			46 (67)		
	Other			6 (9)		
Gender						
	Male		50 (82)	62 (90)	44 (73)	114 (70)
	Female		11 (18)	7 (10)	16 (27)	48 (30)
Age						
	≤ 65 yrs		42 (69)	36 (52)	39 (65)	109 (67)
	> 65 yrs		19 (31)	33 (48)	21 (35)	53 (33)
Tumor site						
	Hypopharynx		10 (16)		8 (13)	8 (5)
	Tonsil		5 (8)		4 (7)	22 (14)
	Base of tongue		9 (15)		3 (5)	5 (3)
	Mobile tongue		6 (10)		5 (8)	26 (16)
	Tongue (CNA)					3 (2)
	Floor of the mouth				4 (7)	9 (6)
	Larynx		18 (30)	69 (100)	33 (55)	61 (38)
		Vocal cord			22 (37)	
		Glottis		45 (65)	2 (3)	
		Supraglottis		11 (16)	4 (7)	
		NA		13 (19)	5 (8)	
	Nasopharynx					10 (6)
	Oral cavity, other		13 (21)		3 (5)	18 (11)
Stage						
	I		6 (10)	40 (58)	23 (38)	60 (37)
	II		12 (20)	9 (13)	11 (18)	20 (12)
	III		14 (23)	10 (15)	9 (15)	18 (11)
	IV		29 (48)	10 (15)	15 (25)	55 (34)
	NA				2 (3)	9 (6)
Alcohol						
	Heavy use			12 (17)		
	Nonheavy use			51 (74)		
	NA			6 (10)		
Tobacco						
	Nonsmoker			2 (3)		
	Smoker			67 (97)		
		<10 cigarettes/day		9		
		10-20		49		
		>20		7		
		NA		2		

CNA = classification not available
NA = data not available

2.2. HPV PCR and genotyping (I, II)

The detection of HPV DNA using SPF10 PCR was done by a microtiter plate-based probe hybridization assay (DEIA) and genotyping by a line probe-based genotyping assay described in detail elsewhere (Studies I and II).[191] In addition, a single-phase PCR with primers FAP59/64[192] and a nested PCR with primers CP65/70 and CP66/69[193] (Study I) were both performed to detect the majority of cutaneous and mucosal HPV types. A nested PCR with consensus primers (Study II) My09/11[194] and GP5+/6+[195] was also performed.[196]

Real-time PCR (Study I) was performed using an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Perkin-Elmer, Wellesley, MA). Amplification and quantification of E2 and E6 were carried out simultaneously in a separate reaction tube.

2.3. Immunohistochemistry (I, III)

HPV L1 structure protein immunostaining (Study I) was conducted to examine L1 structural protein expression in HNSCC. We used HPV-33 L1 monoclonal antibody, which cross-reacts with HPV L1 proteins of most HPV types[197] (a kind gift from M. Sapp, University of Mainz, Germany), with Ventana DAB Kit (Ventana Medical Systems, Inc., Tucson, AZ) in a Ventana Discovery automated ISH-IHC Slide Stainer (Ventana Medical Systems).

For tumor lymphatic and blood vessel density determination (Study III), the antibodies used were a polyclonal rabbit IgG antibody against human LYVE-1[198] (a kind gift from Dr. David Jackson, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK) to detect lymphatic vessels, and a monoclonal mouse anti-CD31 antibody (Novocastra Laboratories Ltd., Newcastle, UK) to detect blood vessels. To determine the blood vessel density (BVD) and the lymphatic vessel density (LVD), the section was first scanned using low magnification, after which two areas (hot spots) showing the highest density of vessels within the tumor or at the tumor margin (in case of lymphatic vessels) were selected.[199] The average vessel count was then determined by counting all immunostained vessels at the two hot spots from one section using a 200 x magnification field corresponding to an area of 0.95 mm^2. Similar vessel counts were obtained with and without using an ocular grid. All vessel counts were assessed without knowledge of the clinical data.

2.4. HLA-DRB1 and -DQB1 sequencing (IV)

HLA-DRB1* (01, 03, 04, 07, 08, 09, 11, 12, 13, 15) and HLA-DQB1* (0201, 0202, 0203, 0301, 0302, 0303, 0402, 0501, 0602, 0603, 0604, 0614) alleles were determined with the Inno-LIPA reversed-dot blot kit (Innogenetics, Dartford, UK). The sequencing was performed at The Finnish Red Cross, Blood Transfusion Service, Helsinki, Finland.

2.5. Statistical analysis (I-IV)

Study I - To investigate dependence between explanatory variables, we performed Fisher's exact test (two-tailed). All *p*-values of less than 0.05 were considered statistically significant. The relationships between survival times and expalanatory variables were studied with the Cox proportional hazards model.

Study II - Fisher's exact test was applied to test the equality of response probabilities in two patient groups. Shifts in age distribution between two age groups were detected using the exact Wilcoxon-Mann-Whitney test with StatXact version 7 (Cytel Inc., Cambridge, MA). Survival of the patients was estimated by the actuarial method and corrected for competing causes of death by estimating relative survival. The relative survival rate is defined as the ratio of observed and expected survival rates of a comparable group of patients from the general population matched by country, sex, age, and calendar time. The patient survival was measured by the cumulative relative survival rate (CR) corrected for heterogeneity in patient withdrawal.[200] Likelihood ratio tests for differences in relative or observed survival rates between patient groups during the entire follow-up period were applied.[201] Life table parameters were estimated and tests conducted using survival package SURV3 version 3.01 (Finnish Cancer Registry, Helsinki, Finland). Proportional hazards models, the hazard ratios (HRs) of death, were obtained[202] with PROC PHREG using statistical software SAS release 8.02 (SAS Institute Inc., Cary, NC). Ties in survival were handled with the exact method. The patients were followed up to the end of October 2005.

Study III - Correlations between CD31 and LYVE-1 vessel counts were calculated with the Spearman rank correlation test and the vessel count was

regarded as a continuous variable. CD31 and LYVE-1 scores between groups were compared with the Mann-Whitney test. Frequency tables were analyzed with chi-square test. Variables that predict the presence of regional metastases were assessed using logistic regression. All *p*-values are two-tailed.

Study IV - Fisher's exact test (2-tailed) was used for Cross-tabulations between different haplotypes. A *p*-value of less than 0.05 was considered significant.

Results and discussion

1. HPV prevalence and viral load in HNSCC (I)

1.1. HPV prevalence and genotypes

Of the 61 HNSCC samples, 37 (61%) were positive for HPV. The most prevalent HPV type was HPV-16 (31/37, 84%). HPV-33 was found in 10/37 positive samples (27%). Multiple infection was observed in 8/37 samples (22%). The frequency of HPV-positive samples varied according to anatomical site: 5/5 (100%) in tonsil, 11/15 (73%) in tongue, 7/13 (54%) in other oral cavity, 9/18 (50%) in larynx, and 5/10 (50%) in hypopharynx. The differences in HPV prevalence between anatomical sites did not reach statistical significance in this material. Only eight samples were HPV-positive (either HPV-16 or -33) with FAP59/64, CP65/70, and CP66/69 primers. No cutaneous types were found.

In line with other studies,[8,160,170] we observed a high prevalence of HPV in tonsillar carcinomas (Fig. 1, Study I), suggesting a causal role of HPV in this subset of HNSCC.[203,204] HPV prevalence in the larynx and hypopharynx was higher in our material than in other studies, where the prevalence in both laryngeal[165,167] and hypopharyngeal[11,157,168] carcinomas was low. This is likely a result of our small patient pool, and therefore, must be interpreted with caution. In this series of Finnish patients, HPV prevalence in laryngeal carcinomas was higher than in our larger prospective laryngeal carcinoma series (Study II), in which heterogeneous material from different Nordic countries was investigated.

1.2. HPV viral load and physical status (I)

HPV viral load and physical status were analyzed in 25 HPV-16-positive samples with an adequate amount of DNA for analysis. A large variation between viral loads in different HNSCC samples was noted. All five tonsillar samples harbored a very high viral load, 46 620 to 4 901 400 copies of E6/10 000

cells, whereas nontonsillar samples harbored only 1 to 677 copies of E6/10 000 cells (Table 2, Study I).

HPV DNA existed in all forms: episomal (n=8), integrated (n=11) and mixed (n=4). Large tumors (T3 and T4) were overrepresented in the episomal HPV DNA group as compared with the integrated (p=0.0181) and mixed (p=0.019) groups. This finding suggests that in these T3-T4 tumors HPV has not been the initiating factor in carcinogenesis; instead the virus may have infected malignant cells already featuring immortality and infiltrative growth.

1.3. Expression of viral capsid protein in HNSCC (I)

HPV L1 protein was not detected in the tissue sections of HPV-positive tumor samples. This was also true for the high viral load-containing tonsillar carcinoma specimens, although our positive controls, cervical intraepithelial neoplasia III and juvenile-onset laryngeal papilloma samples, showed strong expression of L1.

The absence of late capsid protein expression in carcinomas is probably due to synthesis of L1 taking place mainly in productive infections,[205] where epithelial differentiation is seen, and not in carcinomas. Epithelial productive infection requires terminal differentiation of infected cells, which activates the synthesis of capsid proteins. HPV capsid protein expression has previously been detected in premalignant lesions and in keratosis.[205] In carcinomas, the prevalence of capsid protein expression is reported to be low.[206]

1.4. Prognostic significance of HPV status (I)

We found no significant difference in the survival of HPV-positive and HPV-negative patients. HPV status had no effect on survival in the whole series or when analyzed separately for different anatomical sites. This result is probably due to the small number of patients and the relatively short follow-up (mean 24.5 months). Moreover, the study design was not the most suitable for reliable survival analysis. Previous studies have shown better survival for HPV-positive patients. [169,207,208] However, some reports fail to confirm this.[209,210] While HPV may be a marker for better prognosis in tonsillar carcinomas, as suggested by Mellin et al.,[8,169] or in distinct patient groups such as patients with an advanced stage of the disease at diagnosis,[211] HPV DNA may not be prognostic

in all head and cancers. Moreover, lifestyle habits have a strong effect on the survival in the malignancy; Ringström and colleagues[208] noted that HPV-positive patients consumed less alcohol than their HPV-negative peers.

For analysis of the impact of HPV infection on the clinical outcome of head and neck cancer, studies with larger patient pool are needed. A long-term follow-up and an adequate number of patients are required for a reliable survival study.

2. Roles of HPV, tobacco and alcohol in laryngeal carcinomas (II)

2.1. HPV prevalence (II)

No HPV DNA was found with My09/11 and GP5+/6+ primers. The SPF10 PCR hybridization assay revealed three HPV-positive cases; two were positive for the DEIA and not identified by the SPF10 Line probe assay, and one was HPV-16.

HPV DNA was present in only three samples (4.4%), implying that HPV does not play a key role in laryngeal carcinogenesis. Recently, several studies have connected HPV to oropharyngeal and especially tonsillar malignancies, and reports on laryngeal carcinomas show low prevalences of HPV DNA.[9,167,212] Few studies have assessed HPV prevalence in laryngeal premalignant/dysplastic lesions. Fouret et al.[213] found HPV-16 DNA in 6/57 samples in a series of different stages of dysplasia; however, the samples infected with HPV were mostly mild dysplasias. Low HPV prevalence rates in dyplasias were also reported by Gorgoulis et al.[214] However, Azzimonti et al.[205] detected HPV DNA in 56% (28/50) of their series comprising different stages of dysplasia. Nevertheless, their HPV-positive lesions were mostly mild dysplasias.

Situated in the larynx is an epithelial junctional area similar to that in the uterine cervix, which may serve as a potential infection site for HPV. Interestingly, HPV does not seem to have a major role in laryngeal carcinogenesis, despite being the best-known risk factor for cervical carcinoma. The benign lesions laryngeal papillomatosis[215] and genital warts (condyloma accuminata)[38,216] are, however, both caused by HPV types 6 and 11, but HPV

does not have the same promoter role in malignant disease of the upper aerodigestive tract as in cervical carcinoma.

2.2. Factors associated with survival (II)

Tobacco smoking and alcohol consumption are the best-known risk factors for LSCC and are powerful markers of poor clinical outcome. We were unable to distinguish the effect of smoking on individuals from that of drinking in survival analysis because only three patients were nonsmokers. We therefore categorized the patients as heavy (≥ 30 pack years, one pack year = smoking of one pack of cigarettes/day for a period of one year) or nonheavy smokers and compared survival between these two groups, also analyzing the impact of other possible prognostic factors on survival.

The cumulative relative survival (CR) decreased by about 5% per year up to four years of follow-up among all LSCC patients and by more than 10% during the fifth year of follow-up (Figure 1, Study II). We found that heavy drinkers had a worse 5-year CR, 0.38 (95% confidence interval (CI), 0.15–0.71), than nonheavy drinkers, 0.89 (95% CI 0.69–1.01). The difference in relative survival during the entire follow-up between the drinker groups was significant (p=0.01) in favor of the proportional hazards hypothesis. Heavy smokers had a lower 5-year CR, 0.60 (95% CI 0.33–0.86), than those not smoking heavily, 0.92 (95% CI 0.70–1.05), but the difference in relative survival during the entire follow-up was not significant. The relative as well as the observed survival rates were not different during the entire follow-up between the groups of localized and advanced LSCC, patients aged <65 and ≥65 years at diagnosis, and patients with good oral hygiene and poor oral hygiene. The age distributions did not differ between the drinker groups (p=0.23).

The proportion of advanced stage of LSCC was different between heavy and nonheavy drinkers (p=0.01), being higher among heavy drinkers. The proportion of patients with poor oral hygiene was different between heavy and nonheavy drinkers (p=0.02), higher among heavy drinkers. The age- and stage-adjusted hazard ratio (HR) for death associated with heavy alcohol drinking was 3.0, although the confidence interval was wide (95% CI 1.1–8.2). In the same model, the HR for death was also significantly increased, 3.3 (95% CI 1.1–9.6), among LSCC patients at least 65 years at diagnosis compared with

younger patients. The HR associated with an advanced stage of LSCC was nonsignificantly increased, 2.2 (95% CI 0.8–5.7). Neither interactions between age, stage, and heavy alcohol drinking nor adding a variable for heavy tobacco smoking or poor oral hygiene and their interactions with the previously mentioned factors improved the model. The null hypothesis of a multiplicative joint effect of heavy alcohol drinking and heavy tobacco smoking was not rejected.

History of chronic laryngitis, gastroesophageal reflux disease (GERD) or orogenital sex contacts was rare in our LSCC patients; no association was present between these parameters and patient outcome.

Case-control studies have shown the risk for HNSCC to be multiplicative when a patient has a history of both smoking and heavy drinking.[1,2,217] However, few studies exist on nonsmoking alcohol drinkers and laryngeal cancer. Bosetti et al.[136] found individuals consuming over 8 drinks/day to have an increased risk for LSCC (OR 2.46, 95% CI 0.98-6.2), and for nondrinking smokers the risk was far greater (OR 9.38, 95% CI 3.35-26.26). Tuyns et al.[218] observed only a slight increase in relative risk for heavy alcohol use (≥ 80 g ethanol/day). Burch et al.[219] measured lifetime alcohol consumption and found lifetime consumption of over 737 kg alcohol to increase the risk for LSCC (RR 7.7), but this result was obtained from a very small group of patients. Survival reports concerning the effect of smoking and drinking on LSCC are controversial. Crosignani et al.[220] reported alcohol intake not to have an effect on survival, and Pradier et al.[221] showed a similar result with tobacco smoking. Nevertheless, it seems that in LSCC smoking has a stronger effect on survival than alcohol.[147,218,220,222]

Estimate the actual borderline risk of alcohol intake in cancer is difficult. Tobacco smoking is considered hazardous at any level, although reports show differences in risk between heavy and nonheavy smoking and different tar-yield levels. Studies classify alcohol intake as lifelong consumption or absolute alcohol consumed during different time periods. As Polesel[223] criticized, the result of a study can easily be altered by changing the cut-off points for consumption.

3. Blood and lymphatic vessels densities in HNSCC (III)

3.1. Tumor lymphatic vessel density (LVD)

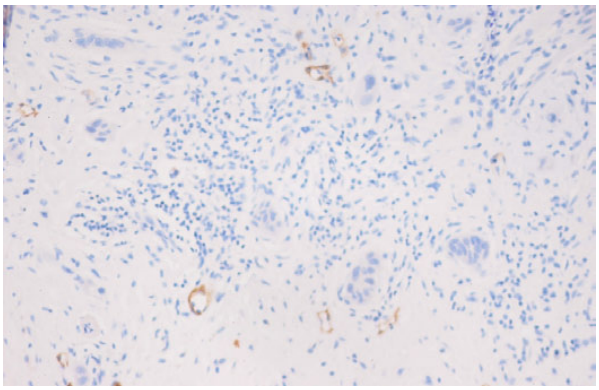
LYVE-1-positive staining was mainly present in thin-walled lymphatic structures and in tissue macrophages. Most positive lymph vessels resided in the peritumoral area or in the tumor front. Twenty-four of the samples (40%) were LYVE-1-negative. The lymphatic vessel densities (LVDs) were investigated by two observers and the agreement was good (Spearman rank correlation coefficient 0.88). Intratumoral lymph vessels were identified in 12 of the 36 cases that showed staining for LYVE-1.

The median tumor LVD was 5/mm^2 (range 0 to 29/mm^2), and high lymph vessel counts were associated with high blood vessel counts (p=0.002, Spearman rank correlation coefficient 0.41). The LVD was not significantly associated with the tumor size (Table 2, Study III, p=0.52), histological grade (p=0.33), or patient's age at diagnosis (p=0.091). Vocal cord carcinomas had a lower LVD than head and neck carcinomas at other sites (Table 2, Study III, p=0.016). Intratumoral lymph vessels were detected at about an equal frequency in vocal cord and other carcinomas (18% vs. 22%, respectively, p=0.75), but peritumoral lymph vessels were less common in vocal cord carcinomas (6/22, 27% vs. 22/38, 58%, p=0.017). Peritumoral LVD was also smaller in vocal cord carcinomas than in the rest of the cancers (median 0/mm^2 vs. 6/mm^2; p=0.026).

3.2. Tumor blood vessel density (MVD)

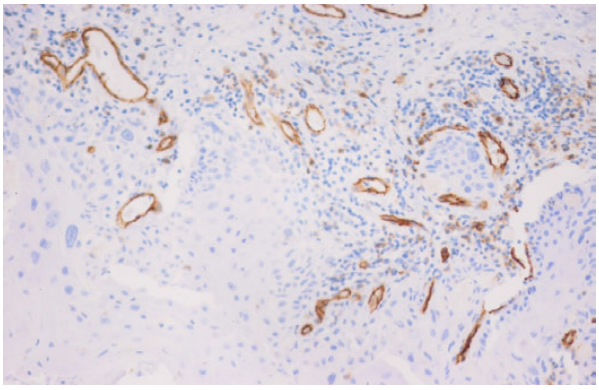
CD31-expressing microvessels were present intratumorally, at the tumor edge, and along the peritumoral rim. Microvessel density was not associated with tumor size (Table 2, Study III, p=0.79), histological grade (p=0.76), or age at diagnosis (p=0.30). The median BVD was smaller in vocal cord carcinomas than in other HNSCCs (Table 2, Study III, p=0.006).

Figure 5.



LYVE-1 immunostaining shows lymphatic vessels in the tumor margin. 200 x magnification.

Figure 6.



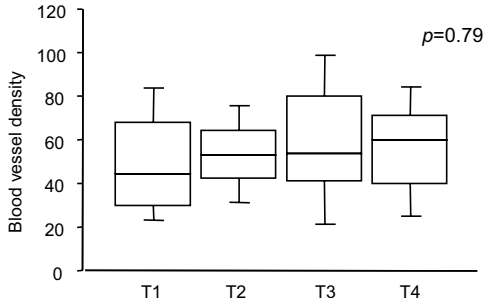
Intra- and peritumoral blood vessels immunostained with CD31 antibody. 200 x magnification.

3.3. Associations of BVD and LVD with regional metastasis

Primary tumor size and tumor location in the vocal cords were the most important single predictive factors for the presence of regional metastases at diagnosis in a logistic regression model (p=0.010 and p=0.020, respectively). Since only one vocal cord carcinoma (5%) had given rise to regional metastasis,

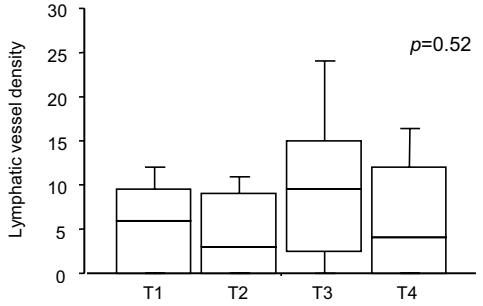
we evaluated the predictive value of BVD and LVD among the rest of the carcinomas (n=38). In this subset, tumor size predicted best the presence of regional metastases (OR 2.2, 95% CI 1.1 to 4.5, p=0.035). Addition of neither BVD nor LVD to the model improved the accuracy.

Figure 7.



Blood vessel density was not associated with tumor size.

Figure 8.



Lymphatic vessel density was not associated with tumor size.

Intratumoral LVDs found in cancers are generally lower[182,185,199,224-228] than peritumoral LVDs[182,199,225,228,229] or LVD of normal structures.[225,230] Recent reports have assessed LVD in HNSCC in general.[182,231] Maula et al.[182] found HNSCC patients with intratumoral lymph vessels to have, a poor outcome but LVD was

not an independent prognostic marker in multivariate analysis. To date, the prognostic significance of LVD remains unclarified. Peritumoral lymphatics have been hypothesized to be key elements in tumor dissemination,[186] but LVD might not be the best way to estimate peritumoral lymphatics since vessel numbers may be smaller, they may be tortuous, and a single vessel may appear several times in one cross-section.[232]

4. HLA-DRB1* and -DQB1* associations in HNSCC (IV)

4.1. Candidate prognostic alleles

Our *a priori* alleles, DQB1*0501 and DR6 (DRB1*13,14), were selected because they were previously reported to have a role in recurrent respiratory papillomatosis (RRP)[26] and HNSCC,[190] and DQB1*0201 because it may be prognostic in RRP and cervical carcinomas.[20,27] In the series of 162 HNSCC patients, our findings on candidate prognostic alleles remained only implicative. HLA-DQB1*03 (DQB1* 0301,0302,0303) was more often found in patients than controls (p=0.051, Fisher's exact test, two-tailed). A protective tendency was observed with allele DQB1*0502, as it was more common in the control group.

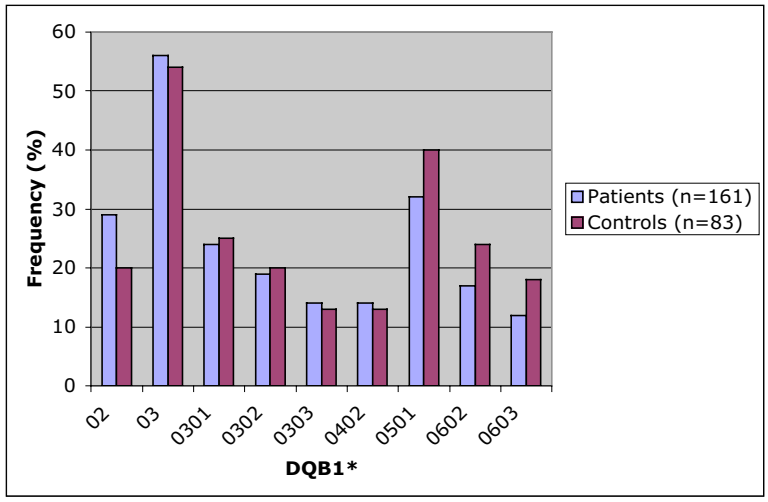
Patients with DRB1*03 more often had a node-negative disease at diagnosis (p=0.036). HLA-DRB1*04 was more frequent in patients with relapse-free disease than in patients who developed a relapse (p=0.058). Patients carrying DRB1*08 or DRB1*13 had smaller tumors at diagnosis than those lacking both (chi-square test likelihood ratio, 0.036 and 0.04, respectively).

We found no disease associations with the DRB1*1501-DQB1*0602 haplotype, the best-known risk allele for cervical cancer,[17] and HLA-DQB1*0501 was also evenly distributed between patient and control groups.

DRB1* alleles were associated with favorable prognostic signs. Apple et al.[19] hypothesized that DRB1* alleles are important in protection against cancer. Although our study did not present significant associations between class II alleles and risk for cancer or disease outcome, potential candidate alleles appear to exist. We did not have tumor samples from patients for HPV DNA analysis.

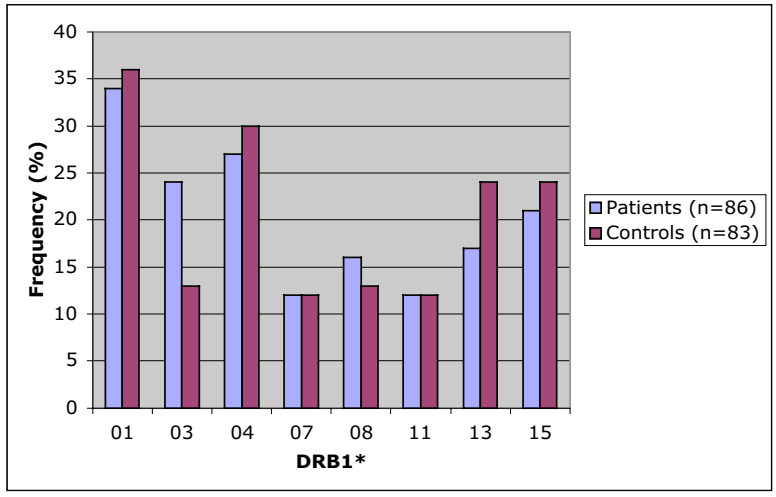
This is a limitation because disease-associated alleles may differ depending on the presence of HPV DNA, as reported for cervical carcinomas.[25]

Figure 9.



HLA DQB1* frequencies in HNSCC patients and controls.

Figure 10.



HLA DRB1* frequencies in HNSCC patients and controls.

Conclusions

Results of our have led to the following conclusions:

I. High-risk HPV DNA is present in a subset of HNSCC. It exists in episomal, integrated, and mixed forms. The viral load is significantly higher in tonsillar specimens than in carcinomas arising from other subsites. Coinfections of HPV-16 and other malignant (HPV-33) and low-risk (HPV-11) types are present in HNSCC, while cutaneous types are rare.

II. HPV DNA prevalence is low in laryngeal carcinomas. This suggests that HPV probably does not play a major role in laryngeal carcinogenesis. Almost all patients in our series were smokers and our study evaluated the role of other potential prognostic factors as well. Heavy alcohol intake predicts poor overall survival in LSCC. Heavy drinkers are also younger at diagnosis. History of chronic laryngitis, gastro-esophageal reflux disease, and frequent oro-genital sex contacts were uncommon in our laryngeal carcinoma patients.

III. In vocal cord carcinomas, the lymphatic and blood vessel densities are significantly lower than in HNSCC arising from other sites. This is in line with the low metastasizing capacity of vocal cord carcinomas. In addition, lymphatic and blood vessel densities correlated strongly with each other in HNSCC. However, neither lymphatic vessel density nor blood vessel density correlated with tumor size or had any prognostic relevance.

IV. HLA class II alleles may have an impact on head and neck carcinogenesis. Certain alleles are associated with good prognostic signs. Moreover, distinct alleles may protect againts or predispose to cancer. Larger studies are, however, required to confirm these findings.

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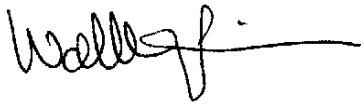
Helsinki, 2006



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