HLA Expression in Head and Neck Squamous Cell Carcinoma

A Profile to Escape?

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HLA Expression in Head and Neck Squamous Cell Carcinoma

A Profile to Escape?

HLA Expressie in Hoofd-Halstumoren

Een ontsnappingsprofiel?

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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FOR IMMUNOLOGY
AND INFECTIOUS DISEASES

“De Boom der Kennis is niet die des Levens”

G.N.G. Byron

Voor Joyce

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Chapter 1

General Introduction

1.1 Head and Neck Squamous Cell Carcinoma

Head and neck squamous cell carcinoma (HNSCC) represent the fifth most common human cancer worldwide. Its global incidence has increased over the past 20 years from 500,000 to 780,000 new cases per year [1, 2]. HNSCC is an aggressive epithelial malignancy which arises from the mucosal membranes of the upper-aerodigestive tract (i.e. pharynx, hypopharynx, and larynx) and the oral cavity. Alcohol and tobacco consumption are the most important risk factors in the development of HNSCC [3-5]. The incidence of HNSCC in males is much higher compared to females due to their drinking and smoking habits. However, more and more females develop HNSCC because of the change in their consumption pattern. Was the proportion between Dutch males and females with a larynx carcinoma in 1970 1 to 23, nowadays it is 1 to 5.5 [6].

In spite of much effort over the past decades in improving diagnosis and treatment, involving surgery, radiation and chemotherapy, the 5-year survival rate for patients with HNSCC is among the lowest of the major cancers. It has remained less than 50% for the last 30 years primarily due to recurrences [7, 8]. Early stage carcinoma can be treated highly effective with surgery or radiotherapy. However, over 70% of the patients present at diagnosis with advanced stage locoregionally disease. Non-operable tumors are treated with chemotherapy, while incurable metastasizing tumors are treated palliative. Patients develop metastatic disease in 15% to 25% of the cases [9]. Moreover, patients develop second primary tumors at an annual rate of 3 - 7% [8]. Tumor stage, localization of the primary tumor and the presence of nodal or distant metastases are currently the only specific prognostic factors that are routinely considered upon treatment considerations. However, HNSCC of the same stage often respond differently to the same treatment indicating heterogeneity in biological behavior.

Genetic factors involved in HNSCC carcinogenesis include several oncogenes like members of the *ras* and *myc* gene family, *int-2*, *hst-1*, *cyclin D1*, EGF receptor and *Bcl-2* [10]. Genetic alterations involving tumor suppressor genes *p16* and TP53 are frequently observed in HNSCC [11, 12]. Loss of chromosomal regions on 3p, 5q, 6p, 8p, 9p, 9q, 11p, 15q and 17p was often observed in HNSCC and has been associated with the development and progression of the disease [13-15]. However, the exact molecular mechanisms underlying tumor development and the variation in biological behavior of HNSCC are not well understood.

Due to the lack of any significant progression in the treatment of HNSCC to improve patient survival, the development of immunotherapy has gained major interest over the past years [16-20]. Effective immunotherapy is dependent on the identification of suitable tumor associated antigens (TAA) [21]. Therefore, immunotherapeutic interventions aim to initiate and/or augment immunological responses against such TAA. A major obstacle on the road to successful treatment

is the fact that HNSCC are poorly immunogenic and strongly immune suppressive [22-27].

Various mechanisms responsible for the escape of tumor cells from immune recognition have been identified. These include deficient presentation of immunogenic antigens by human leukocyte antigen (HLA) molecules, defects in antigen processing, inadequate costimulatory interactions, production of immunosuppressive factors, and the lack of sufficient, functional immune cells [28, 29]. It has been shown that the tumor-infiltrating lymphocytes in HNSSC are dysfunctional [30]. There is also evidence that tumor induced factors or serum factors contribute to a decrease in both local and systemic immune responses in HNSCC patients. Among these are prostaglandin PGE_2 which inhibits lymphocyte proliferation, polypeptides such as p15E and the cytokines TGF-β, IL-10, GM-CSF as well as nitric oxide [26, 31-38]. Soluble factors in sera of HNSCC patients, which have not yet been fully characterized, might inhibit lymphocyte proliferation and generation of lymphokine-activated killer cells from peripheral blood lymphocytes [39, 40]. Besides, circulating immune complexes in the sera of these patients were elevated and may contribute to immune suppression via downregulation of natural killer (NK) cell mediated tumor lysis [41]. HNSCC are able to induce functional defects and apoptosis in immune effector cells as well as antigen presenting cells (APCs) by various mechanisms [25, 42-46]. Membranous microvesicles containing biologically active FasL may be involved in the lysis of Fas-positive T cells in the peripheral circulation [47]. Moreover, a decreased number of myeloid dendritic cells in the peripheral circulation and an induction of immunosuppressive immature dendritic cells or CD34+ progenitor cells in the presence of HNSCC has been described [48-51]. These various mechanisms hinder the cellular effectors of the immune system in their efficacy to elicit a tumor directed immune response.

Not only can HNSCC interfere with the cells of the immune system, they also have developed mechanisms to evade or hide themselves from the host. HNSCC frequently have reduced expression of costimulatory molecules, alterations in the HLA class I associated antigen-processing machinery or downregulated HLA class I expression [52-56]. The presentation of TAA by tumor cells on their cell surface through HLA is of crucial importance in immune surveillance and immunotherapy. It presents the only way by which the immune system or immunotherapy can recognize infected or neoplastic cells and eliminate them. In many carcinomas, antigen processing and presentation by HLA is disturbed [15, 57-60]. Aberrant HLA expression is defined by HLA loss-phenotypes: i) phenotype 1, total HLA loss; ii) phenotype 2, HLA haplotype loss; iii) phenotype 3, HLA locus loss, iv) phenotype 4, HLA allelic loss; v) phenotype 5, compound loss [61]. An illustration of each HLA loss-phenotype is depicted in Figure 1. Characterization of HLA loss-phenotypes in tumors of different origin demonstrated that HLA aberrations

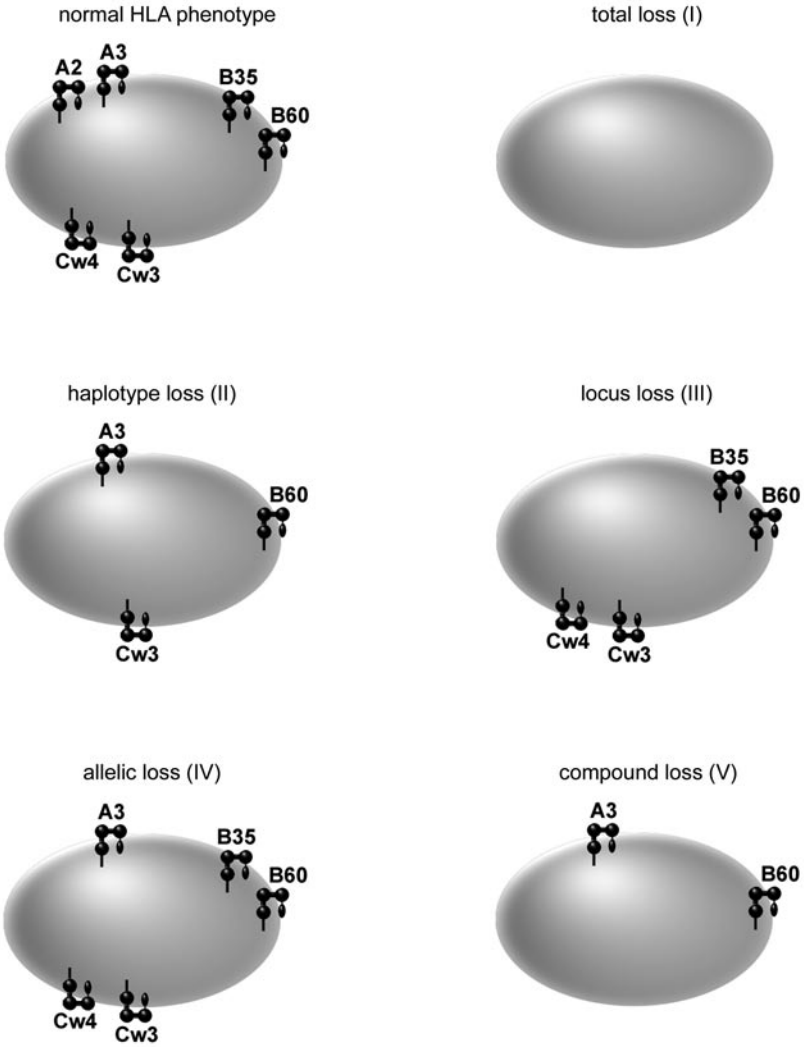

Figure 1. HLA class I tumor phenotypes. The full expressed HLA phenotype is HLA-A2, -A3; HLA-B35,-B60; HLA-Cw4, -Cw3. The total loss phenotype (I) may result from defects in β2m and TAP synthesis, whereas haplotype loss (II) is caused by genomic loss of (parts of) one chromosome 6 by chromosomal non-disjunction, mitotic recombination or chromosomal deletion. HLA locus loss (III) is thought to result from locus-specific transcription downregulation and homozygous deletions, while allelic loss (IV) is caused by mutations and deletions in individual HLA alleles. Compound loss (V) reflects any combination of loss phenotypes.

in HNSCC are distinct compared to melanoma, breast and colon carcinoma [62]. This may reflect that different mechanisms in each tumor type are involved in HLA expression-loss. By identifying the HLA aberrations in HNSCC and studying their underlying molecular mechanisms, effective immunotherapies can be developed that target and eradicate tumor cells of primary lesions and metastases. This would improve the treatment of patients around the world for this aggressive and often life threatening malignancy.

1.2 The Human Leukocyte Antigen Complex

The human major histocompatibility complex (MHC), also known as the human leukocyte antigen (HLA) complex, is contained within approximately 4 Mbp of DNA which accounts for ~1% of the human genome. It is located on the short arm of chromosome 6 at 6p21.3 of which an overview is shown in Figure 2. This gene cluster contains many functional interrelated genes which have immunological functions in antigen processing and presentation. The HLA cluster is divided into the class I, class II and class III region, reviewed in [63].

The HLA class I region contains the highly polymorphic classical HLA genes

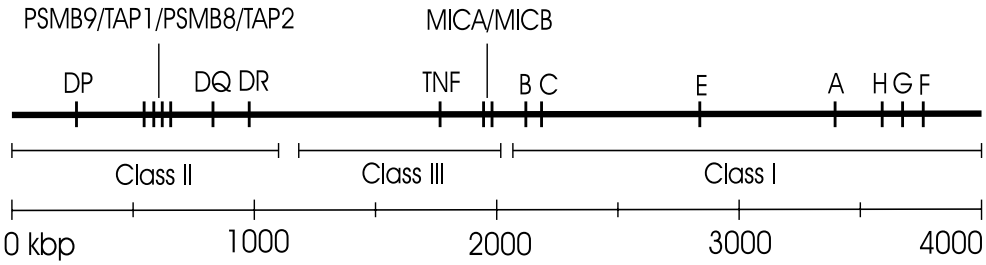

Figure 2. Schematic overview of the HLA region on chromosome 6p21.3 indicating the location of the HLA genes and functional related genes. The HLA class II region is located centromeric, whereas the class I region is located telomeric. The ruler indicates the relative distance between the genes in kilo basepairs (kbp).

HLA-A, HLA-B and HLA-C, and the less polymorphic non-classical HLA genes HLA-E, HLA-F, HLA-G and HLA-H. Each gene encodes an α-chain of a class I molecule. In the endoplasmic reticulum (ER), the HLA α-chain forms a protein complex with β2 microglobulin and an antigenic peptide which is crucial for stable complex formation. This HLA class I antigen complex is translocated to the cell surface to present the antigenic peptide to CD8+ cytotoxic T cells which can elicit a cellular immune response [64]. HLA class I antigen molecules are expressed on most nucleated cells to facilitate immune surveillance.

The main surface-expressed HLA class II molecules are HLA-DR, HLA-DQ and

HLA-DP. Each class II molecule consists of two transmembrane glycoproteins, the α-chain and β-chain, that are encoded by class II α and β genes located in the HLA class II region. In general, HLA class II molecules are expressed on professional antigen presenting cells, i.e. macrophages, monocytes, B cells and dendritic cells that, amongst others, present antigenic peptides to CD4+ T cells to mount a humoral immune response [65]. In the HLA class II region, genes encoding for proteasome subunit β (PSMB) 8, PSMB9, transporter associated with antigen processing, TAP1 and TAP2, are located. These molecules are involved in the HLA class I antigen processing.

The HLA class III region is interspersed between the class I and class II region and contains a large number of genes that are likely to be involved in the immune and inflammatory responses [63, 66, 67]. However, genes in the class III region have also been implicated in a number of non-immune-related diseases such as congenital adrenal hyperplasia and sialidosis, which is a rare inherited metabolic disorder that belongs to a group of diseases known as lysosomal disorders [67].

In general, HLA class I molecules present endogenously derived peptides, while HLA class II molecules present exogenously derived peptides on the cell surface [68]. In this way, infected or malignant cells can be eradicated by cytotoxic T cells via presentation of viral peptides or TAA by HLA class I molecules. By presenting exogenously derived peptides (e.g. bacterial/viral peptides) via HLA class II molecules to CD4+ T cells, a humoral immune response can be mounted to clear the host of the invading pathogens. However, it has become evident that this functional distinction between HLA class I and class II is ambiguous. Cross-presentation of exogenous peptides by APCs, priming CD8+ cytotoxic T cells, was first extensively described in the mid-1970s and has since been the focus of many studies [69-72]. The precise functioning and consequences of cross-presentation are not yet known. In spite of this functional ambiguity, it is currently believed that recognition and eradication of neoplastic cells by immune cells is mainly facilitated via HLA class I antigen expression.

1.3 HLA Class I Antigen Processing and Assembly

Antigen processing of HLA class I presented peptides is a crucial process, as the nature and immunogenicity of the peptide determines whether a CD8+ T cell response can be evoked. The majority of the peptides are generated from endogenous proteins by the antigen processing machinery. HLA class I antigen processing involves three main steps: cytosolic peptide generation by protein degradation, peptide transport into the ER and peptide assembly with HLA class I molecules [73-75]. A schematic overview of HLA class I antigen processing is depicted in Figure 3.

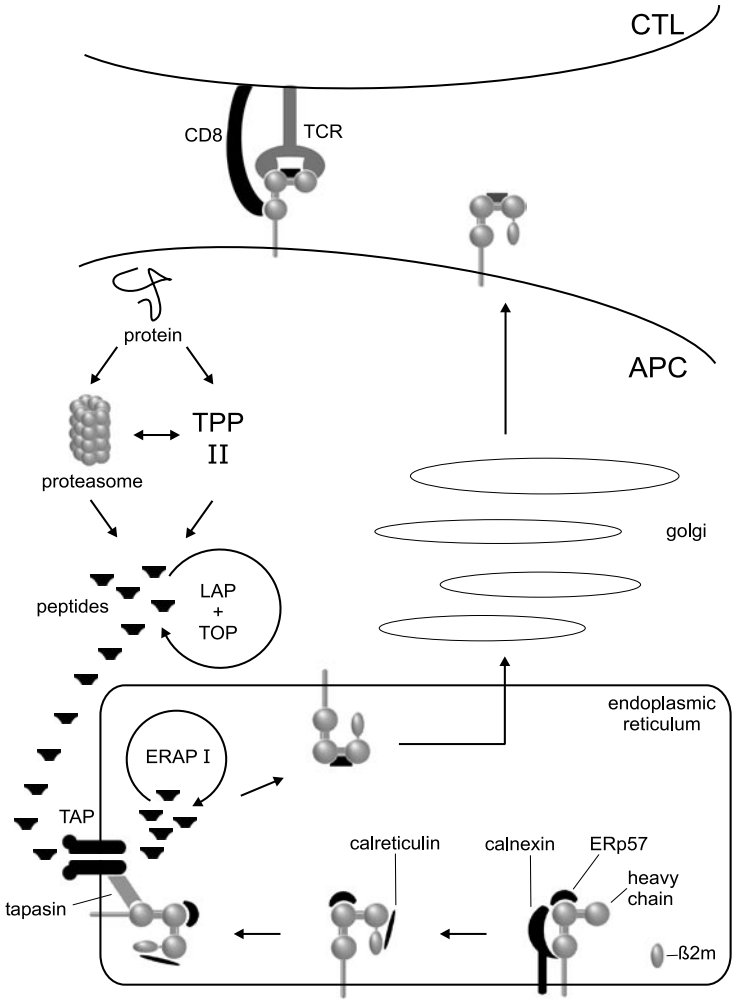

Figure 3. HLA class I antigen processing and presentation to cytotoxic T lymphocytes (CTL) in an antigen presenting cell (APC). Ubiquitinated proteins are degraded by the proteasome and/or tri-peptidyl peptidase II (TPPII), leucine aminopeptidase (LAP) and thimet oligopeptidase (TOP) into peptides. These peptides are translocated into the endoplasmic reticulum (ER) by transporter associated with antigen processing TAP. In the ER, HLA class I/β2m complexes are formed. This process is chaperoned by endoplasmic reticulum protein 57 (ERp57), calnexin and calreticulin. The peptides are modified by ER aminopeptidase 1 (ERAPI) and bound by HLA class I/β2m complexes which is chaperoned by tapasin. The stable HLA class I/β2m/peptide complexes are subsequently transported through the Golgi to the cell surface. There, the peptides are presented for recognition by cytotoxic CD8+ T cells through the T cell receptor (TCR).

Ubiquitinated endogenous proteins, including potential TAA in tumor cells, are degraded by proteases into peptides. The C-terminus of the peptides is generated by the proteasome [76]. This is a large multi-catalytic protease complex consisting of a 19S regulatory complex and the 20S core proteasome which contains four heptameric α and β rings. Figure 4 shows an illustration of the 20S core proteasome and the functionally related 20S core immunoproteasome. The two outer α rings of the 20S core proteasome have structural and regulatory functions while the two inner β rings harbor the catalytic center. The proteasome subunits β (PSMB) 5, PSMB6 and PSMB7, mainly determine the efficacy to produce antigenic peptides capable of binding HLA class I antigens [77]. They preferentially cleave after hydrophobic amino acids (chymotrypsin-like activity) and amino acids with basic residues (trypsin-like activity) or acidic residues (peptidyl glutamyl peptide hydrolyzing activity) [78]. Upon interferon-γ (IFN-γ) release, PSMB5, PSMB6 and PSMB7 are substituted by PSMB8, PSMB9 and PSMB10, respectively [79]. This complex is also known as the immunoproteasome which has altered catalytic properties compared to the proteasome. The cleavage after hydrophobic and basic amino acids is enhanced by PSMB9 in contrast to the cleavage after acidic amino

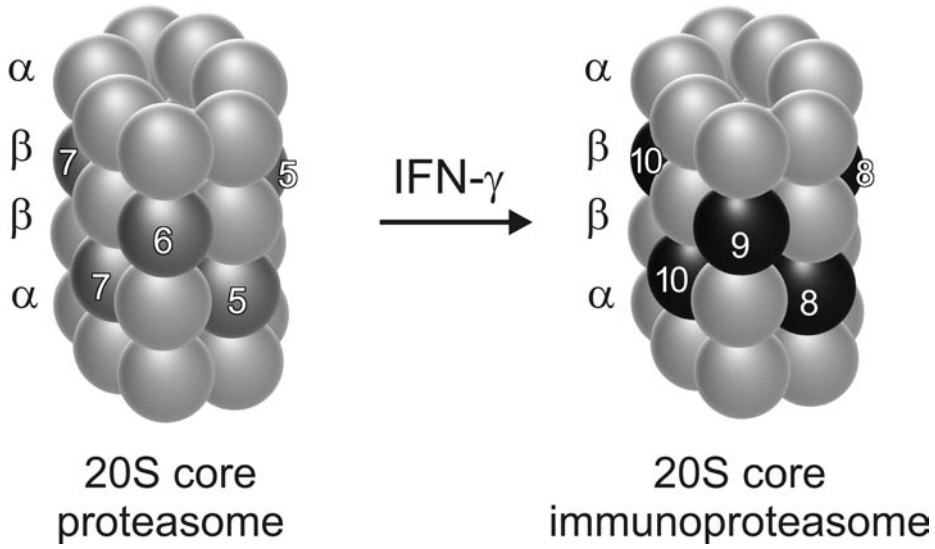

Figure 4. The schematic structure of the 20S core proteasome with PSMB5, PSMB6 and PSMB7 in the inner two β rings. Under IFN-γ upregulation, these subunits are exchanged for PSMB8, PSMB9 and PSMB10 respectively, which enhances the cleavage of ubiquitinated proteins after hydrophobic and basic amino acids at the C-terminus. This improves peptide binding in the HLA class I groove.

acids, which is downregulated by PSMB8 [80, 81]. The function of PSMB10 is not yet known. The generated peptides are more suitable for TAP transport and binding to the HLA class I peptide binding groove. This enhances the antigen presentation and subsequently the immune surveillance. The 19S regulatory complex recognizes ubiquitinated proteins by its ubiquitin (Ub) receptor, unravels the protein structure in an ATP-dependent manner and feeds the protein to the 20S core proteasome after cleavage of the polyUb chains by isopeptidase activity. A substitute for the 19S regulatory complex is the proteasome activator PA28 protein which enhances the peptide generation of the core proteasome by facilitating dual cleavage [82]. Recently, it has been shown that the novel C-terminal cleaving protease tri-peptidyl peptidase II (TPPII) is also involved in antigen processing [83-86]. It is suggested that TPPII functions as a proteasome analogue but can also substitute proteasome functions [87, 88]. Moreover, it is known that TPPII generates unique peptides [85, 86]. For example, the HIV-Nef (73-82) epitope is only generated by TPPII and not by the proteasome [83]. Therefore, TPPII is an important new player in the antigen-processing machinery which gains more and more attention.

After C-terminal cleavage by the proteasome and TPPII, the oligopeptides are processed at the N-terminus by aminopeptidases like leucine aminopeptidase (LAP) and thimet oligopeptidase (TOP) [89]. These proteolytic processes, required for antigen presentation, form a delicate balance between generating and destroying epitopes [90]. LAP is an IFN-γ inducible, zinc-binding metallopeptidase which can trim multiple HLA class I ligands. It may therefore play an important role in the production of antigenic epitopes. TOP is a soluble thiol-sensitive metallopeptidase that cleaves internal bonds in peptides (endopeptidase). It has been reported that overexpression of TOP destroys antigenic peptides and limits the extent of MHC class I antigen presentation [91]. Two other peptidases involved in HLA class I antigen processing are bleomycin hydrolase (BH) and puromycin-sensitive aminopeptidase (PSA) [92]. These are cytosolic N-terminus cleaving peptidases which degrade the minimal epitope efficiently to smaller peptides, so that these enzymes may also play a role in general protein turnover and help recycling peptides as components for protein synthesis [92, 93].

After cleavage in the cytosol by the variety of peptidases, the antigenic peptides are translocated into the ER by the heterodimeric transporter associated with antigen processing which consist of a TAP1 and a TAP2 protein [94]. TAP1 and TAP2 are members of the ATP-binding cassette (ABC) family. Binding of a peptide to the TAP complex induces a conformational change in the TAP-peptide complex which opens a pore into the ER lumen by ATP hydrolysis [95]. In this way, peptides, of up to 40 amino acids, are transported from the cytoplasm into the lumen of the ER. In the ER, the antigenic precursor peptides are trimmed by ER aminopeptidase 1 (ERAP1) to epitopes of 8-9 residues [96, 97]. ERAP1 can trim peptides, before they bind the

HLA class I molecules, to optimally fit the HLA class I molecules [96]. However, it has also been shown that ERAP1 can limit the antigen presentation by destroying nine-residue peptides [98]. Beside ERAPI and signal peptidases, no other known peptidases in the ER have been demonstrated convincingly [99].

The assemblage of the HLA class I heavy α chain, the β2 microglobulin light chain and the peptide in the ER is a well orchestrated process involving several chaperone proteins [73, 75, 100]. The HLA class I heavy chain binds with calnexin for protein stability. After binding of β2 microglobulin, calnexin is substituted for calreticulin which is essential since absence of calreticulin will cause peptide-loading defects. This protein complex associates with chaperone proteins endoplasmic reticulum protein 57 (ERp57) and tapasin which forms a bridge between the HLA class I peptide-loading complex and TAP. After loading of a peptide into the HLA class I binding groove, the HLA class I complex is stabilized and released from the peptide-loading complex to be translocated via the Golgi complex to the cell surface.

1.4 HLA Class I and Cytotoxicity

Cytotoxic CD8+ T cells recognize the peptide in the stable HLA class I/β2m/peptide complex by their T cell receptor (TCR) [101, 102]. Healthy cells present a sampling of their protein content via the so called "self-antigens" for survey by cytotoxic T cells. An infected or malignant cell contains an altered subset of proteins like viral or altered self-proteins and will present parts of these in the form of "foreign-peptides", including potential TAA. CD8+ T cells recognizing self-antigens are deleted in the thymus before they become activated [103-105]. However, foreign-peptides and TAA are no target in this negative selection procedure. The naïve CD8+ T cells that recognize the foreign-peptides and TAA can be activated by mature dendritic cells and elicit a cytotoxic response, killing the infected or malignant cells.

Natural killer cells can also interact with other cells via HLA class I molecules. As the name suggests, these cells are natural killers and eradicate cells with a defective or non-autologous HLA class I expression without prior sensitization. This was postulated as the "missing self" concept [106]. As the former mechanism is important in tumor immunology, the latter is of interest in transplantation [107]. In principle, NK cells provide a first line of defense, but also work as a back-up for the immune system when the cytotoxic T cells are not effective due to the loss of HLA class I expression [108]. NK cells have two distinct families of NK cell receptors [109]. The first family contains the killer cell Ig-like receptors (KIR) that specifically recognize HLA-A, HLA-B and HLA-C alleles on the target cell surface. The second family includes type II molecules characterized by a C-type lectin domain which are expressed as heterodimers composed of a CD94 glycoprotein and a NKG2 family protein. The CD94/NKG2 receptor monitors the overall HLA class I antigen

expression by HLA-E which binds leader peptides derived from HLA-A, HLA-B, HLA-C and HLA-G polypeptides [110, 111]. Depending on the NK cell receptor, activating and/or inhibitory signals are generated, leading to lysis or inhibition of lysis respectively [112]. NK cells also interact with MICA on the target cell of which the expression is stress induced. It is currently thought that MICA binding by NK cell receptors stimulates NK cell mediated lysis. Whether an NK cell will lyse a target cell depends on the balance between inhibitory and activating signals. NK cells not only lyse cells with total HLA class I expression-loss, but also cells with downregulated HLA class I expression [113]. Moreover, *de novo* synthesis of non-classical HLA class I and HLA class II molecules protects tumor cells from NK-mediated lysis [114].

Cytotoxic T cell and NK cell immunity represent two complementary arms of the cellular immune response. Tumor cells may evade a CD8+ T cell response by HLA class I expression-loss, but have to watch their back for NK cells that eliminate cells with HLA class I expression-loss or downregulation. Therefore, the immunologic force of both cellular immune responses may drive tumor cells into an "immune-escape window".

Immunotherapy as a treatment for cancer patients is becoming reality. Clinical immunotherapy trials have been evaluated in melanoma patients, demonstrating that the clinical outcome is not always successful and varies between patients [115-118]. Although many mechanisms may underlie this variation of outcome, TAA presentation by HLA antigens on the tumor cell is one of the most elementary factors. Therefore, detailed characterization of TAA processing and HLA expression in the tumor as well as in metastases is crucial for effective applicability of immunotherapy.

1.5 Outline and Scope of the Thesis

Treatment of patients with HNSCC has not improved considerable over the past decades and is only effective in early stage carcinoma. At diagnosis, most patients present with advanced stage tumors. Immunotherapy has gained interest over the last years to improve disease-free survival of patients with HNSCC. One of the hurdles to overcome is the poor immunogenic and strong immune suppressive properties of HNSCC. Apart from the capability of these tumors to interfere with the functionality of the immune system, they possess mechanisms to become undetectable for the immune system. HLA class I expression and associated antigen processing are elementary mechanisms in cellular immune surveillance and are critically important in the detection and eradication of neoplastic cells. The scope of this study was to include identification and characterization of defects in HLA class I expression on the cell surface and HLA class I associated antigen processing that may play a role in the evasion of HNSCC from immune surveillance.

We investigated the expression of β2 microglobulin, HLA class I and class II in HNSCC using immunohistochemical staining (Chapter 2). HLA class I and β2 microglobulin expression in tumor tissue was frequently affected. Remarkably, almost 50% of the HNSCC showed HLA class II expression.

In β2 microglobulin expression-loss phenotypes, loss of heterozygosity (LOH) has been frequently demonstrated by microsatellite marker analysis. We evaluated the value of β2 microglobulin LOH analysis to define β2 microglobulin expression variants. In HNSCC, microsatellite marker LOH analysis was not reliable to determine β2 microglobulin loss of heterozygosity (Chapter 3).

Fluorescent *in situ* hybridization (FISH) analysis of a selection of 11 HNSCC indicated that β2 microglobulin LOH analysis could not distinguish loss from gain (Chapter 4). Moreover, chromosome 15 aneuploidy, and therefore the β2 microglobulin gene copy number, was complex and heterogeneous within the tumors.

FISH analysis of chromosome 6 in nine selected HNSCC demonstrated that LOH analysis of the HLA region was not representative for genomic loss of HLA (Chapter 5). LOH analysis could not distinguish genomic loss from gain because chromosome 6 aneuploidy was complex and heterogeneous. This provided the proof that LOH analysis is not conclusive and can not predict HLA genomic loss and cell surface expression.

To determine the HLA expression-loss in HNSCC, we investigated HLA-A and HLA-B cell surface expression at the allelic-level in fresh tumor tissue of 15 HNSCC patients by immunohistochemical staining (Chapter 6). Allele-specific HLA loss was demonstrated in approximately 50% of the tumors which would have remained undetected with locus-specific or monomorphic HLA antibodies. HLA typing was required to select allele-specific mAbs. We concluded that HLA expression should

be determined at the allele-specific level to improve our understanding of HLA expression-loss in tumors.

The processing of TAA and the translocation of these peptides by TAP into the ER is as important for immune surveillance as HLA expression. To determine whether TAP polymorphisms are involved in the development of HNSCC by influencing the transport of TAA into the ER for HLA class I antigen presentation, we developed a high-throughput SNaPshot™ assay, to type the TAP1 and TAP2 alleles (Chapter 7). No statistical significant difference was found between the HNSCC panel and the control panel.

Gene expression of the HLA class I associated antigen-processing machinery components was studied by quantitative PCR (Chapter 8). Defects in the antigen processing machinery (APM) gene expression results in a sub-optimal or even loss of TAA processing which contributes to the evasion of tumor cells from immune surveillance. We found a significant upregulation of PSMB7, PSMB8, PSMB9, TAP1 and tapasin, whereas TPPII gene expression was significantly downregulated.

Our findings are discussed in the context of tumor escape from immune-surveillance and rationalized in relation to a potential treatment by immuno-therapeutical mediators (Chapter 9).

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Chapter 2

A Variety of HLA Phenotypes in Head and Neck Squamous Cell Carcinoma Patients Identified with the Workshop Antibodies as Defined in the HLA and Cancer Component.

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Abstract: The frequency of HLA class I antigen loss or down-regulation in head and neck squamous cell carcinoma (HNSCC) was assessed according to the procedure described by the HLA and Cancer Component of the 13th IHWS. Formalin fixed tissue sections of 102 HNSCC were stained with monoclonal antibodies HC-10 (HLA-B & HLA-C) and HC-A2 (HLA-A), anti-β2m antibody L368, and the anti-HLA class II antibody LGII-612.14. Immunohistochemical staining results were analysed and evaluated by two independent observers. The results show that in the majority of tumors β2m is affected in some degree. HC-A2 staining was less frequently positive than HC-10 staining, implying that HLA-A expression is more frequently lost than HLA-B/C expression. HLA class II expression was found in almost 50 percent of the tumors, indicating that HLA class II is likely involved in HNSCC development.

HLA 2004: Immunobiology of the Human MHC. IHWG Press, 2005.

2.1 Introduction

Abnormalities in HLA class I expression are frequently observed in malignancies resulting in an array of HLA class I loss-phenotypes [1-4]. Different HLA class I loss-phenotypes may play a role in the clinical course of a disease and may even affect T cell-based immunosurveillance [2, 5-9]. The potential success of immunotherapy is likely to be abrogated in loss-phenotype disease [10, 11]. HLA loss-phenotypes can be characterized and studied using immunohistochemical staining. As part of the HLA and Cancer Component of the 13th IHWS, the frequency of HLA class I antigen loss or down-regulation in primary and metastatic lesions in various types of malignancies was assessed. We studied the expression of HLA antigens and β2m in primary lesions of head and neck squamous cell carcinoma (HNSCC) by staining formalin fixed tissue sections with monoclonal antibodies HC-10 (HLA-B & -C), HC-A2 (HLA-A), anti-β2m antibody L368, and anti-HLA class II antibody LGII-612.14. A total of 102 HNSCC were analysed and evaluated by two independent observers. Classification of the immunohistochemical staining was divided into positive, negative and heterogeneous patterns as defined by 13th IHWS protocols.

2.2 Materials and Methods

The use of patient materials in this study was approved according to the guidelines of the METC protocol #96/267. Immunohistochemical staining was performed on 4 μm thick paraffin sections of HNSCC lesions. All sections were mounted on silan-coated glass slides and stored overnight at 60°C. Heat-induced epitope retrieval in a 10 mM tri-sodium-citrate-2-hydrate buffer of pH 6.0, prior to immunohistochemical staining, was necessary for all antibodies. The antibodies were diluted in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.2% sodium azide. The HC-10 antibody was used at a dilution of 1:25, HC-A2 at 1:10, L368 at 1:200 and the LGII-612.14 antibody at 1:400. For immunohistochemical staining, the three-step biotin-streptavidin-peroxidase technique was used with DAB as chromogen. Sections were analysed by two independent observers. Any discrepancies were re-evaluated in the presence of a third observer.

2.3 Results and Discussion

During immunohistochemical data analysis, it appeared that one “heterogeneous classification” was too limited to encompass the diverse array of heterogeneous staining patterns in the tumor tissues. We therefore expanded this classification scheme and categorized the immunohistochemical staining patterns of the antibodies as follows: “positive” for positive staining of the tumor (Figure 1A); “weak positive” for weak diffuse staining of the tumor (not shown); “heterogeneous” when tumor staining was partly positive/partly negative (Figure 1B); “negative with positive fields” when the tumor was mainly negative with some small positive fields (not

shown); "positive with negative fields" when the tumor was mainly positive with some small negative fields (not shown); and "negative" for no staining of the tumor (Figure 1C). Negative staining of the tumor was only assigned when some stromal tissue or infiltrating cells were positive, which served as a staining control.

Results of the immunohistochemical staining are depicted in Table 1. Antibody L368, which selectively stains β2 microglobulin, showed that 18% of the tumors totally lost β2m expression on the cell surface. In these tumors, HLA class I cell surface expression (defined by staining with HC-A2 and HC-10) was also lost, except for 2% of the tumors which were negative with positive fields. However, 43% of the tumors showed normal positive β2m expression, leaving 57% in which β2m expression was affected. Thus, in the majority of the tumor panel, β2m expression was affected to some degree.

HC-A2 staining showed that only 5% of the tumors had normal positive staining. Strikingly, 44% of the tumors lost all (26% HC-A2 negative) or the majority (18% HC-A2 negative with some small positive fields) of their HLA-A expression. In contrast, only 22.5% of the tumors (14.5% HC-10 negative and 8% HC-10 negative with some small positive fields) were nearly completely HC-10 negative while 25.5% of the tumors were positive (Table 1). These data suggest that HLA-A cell surface expression is more severely affected than HLA-B/C expression in these tumors. One possible explanation for this is that the more telomeric located HLA-A locus is more susceptible to genomic damage, due to telomeric instability, than the more centromeric located HLA-B/C loci. However, HC-10 stains both HLA-B and

Table 1. Immunohistochemical staining results

	Staining Results (%)					
Antibody	Positive	Weak positive	Negative	Heterogeneous	Negative with positive tumor fields	Positive with negative tumor fields
L368	43	13	18	19	2	5
HC-A2	5	4	26	41	18	6
HC-10	25,5	6	14,5	35	8	11
LGII-612.14	9	8	55	21	4	3

A total of 102 tumor samples were evaluated. The staining pattern of the antibodies was categorized as follows: "positive" for positive staining of the tumor; "weak positive" for positive diffuse staining of the tumor; "negative" if there was no staining of the tumor; "heterogeneous" if the tumor's staining pattern was partly positive, partly negative; "negative with positive fields" if the tumor was mainly negative with some positive fields; "positive with negative fields" if the tumor was mainly positive with some negative fields.

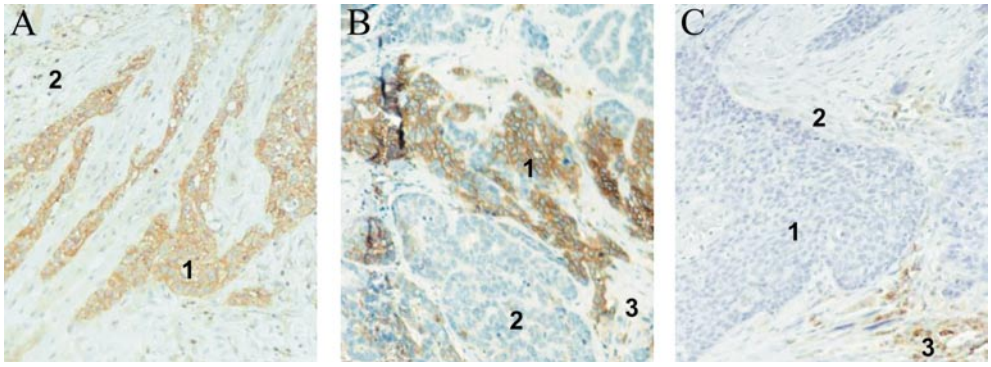

Figure 1. Immunohistochemical staining of HNSCC lesion sections showing A) positive HC-10 staining (50x) of the tumor cells in brown (1), with negative staining of the surrounding stromal cells (2); B) heterogeneous LGII-612.14 staining (50x) with partly positive (1) and partly negative (2) tumor cells. The surrounding stromal cells are negative (3); C) negative HC-A2 staining (50x). No staining is seen in the tumor cells (1) and the stromal cells (2). Positive staining of infiltrating cells acts as a positive control (3).

HLA-C, thereby diminishing the chance of showing a negative staining. Therefore, individual loss percentages of HLA-B or HLA-C expression might be higher than detected by HC-10. HLA-B and HLA-C locus specific antibodies are necessary to study this in more detail.

HLA class I cell surface expression, as studied by HC-A2 and HC-10, is heterogeneous in 41% and 35% of the tumors, respectively. In the majority of tumors, HLA class I expression is not equally affected throughout the tumor. Since tumor development is a very dynamic process, few tumors will be found with a HLA total-loss phenotype. Both HC-A2 and HC-10 staining were negative in only 10% of the tumors (data not shown). Haplotype-loss phenotypes and allelic-loss phenotypes are intermediate stages in which the majority of individual tumor cells will persist. To understand tumorigenesis and loss of HLA in more detail, the use of locus specific antibodies is insufficient. Allele-specific antibodies will be necessary to characterize the HLA haplotype- and allelic-loss-phenotypes. Therefore, HLA typing is required. It should also be noted that HC-A2 and HC-10 do not solely stain HLA-A and HLA-B/C respectively since allelic cross-reactions have been noted [12, 13]. This strengthens the argument that more effort should be directed to identifying allele-specific antibodies in order to characterize and understand HLA class I cell surface expression in more detail.

HLA class II expression was studied by LGII-612.14 staining. Normally, only professional antigen presenting cells (APCs), such as B cells, dendritic cells and macrophages, express HLA class II. However, only 55% of the tumors were completely negative for LGII-612.14 staining, leaving 45% of the tumors showing

some degree of HLA class II expression. It is known that HLA class II expression can be induced by infiltrating inflammatory cells. Nevertheless, our data show that HLA class II cell surface expression is independent of HLA class I expression. Whether the simultaneous expression of both HLA class I and class II molecules on tumor cells affects each others antigen presenting function and thereby perturbs appropriate T-cell immunesurveillance remains to be determined. This study indicates that HLA class II expression in tumors can not be overlooked.

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Chapter 3

The Value of Loss of Heterozygosity of Short Tandem Repeat Markers to Define Expression Variants of the β2 Microglobulin gene.

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Abstract: Short tandem repeat (STR) microsatellite markers are a powerful tool to study loss of heterozygosity (LOH) of a genomic region due to their high degree of polymorphism and their abundance throughout the genome. We studied 53 patients with head and neck squamous cell carcinoma (HNSCC) and correlated LOH data with β2m expression, studied by immunohistochemical staining. The positions of STR markers in relation to the β2m gene vary considerably in different STR marker databases. Therefore, the positions of these STR markers were re-evaluated. Our results indicate that in the majority of HNSCC samples a large region surrounding β2m is affected by LOH. However, the use of STR markers to study LOH within a genomic region is not conclusive, and data from other techniques, such as fluorescence *in situ* hybridization (FISH), are needed.

HLA 2004: Immunobiology of the Human MHC. IHWG Press, 2005.

3.1 Introduction

Loss of HLA expression is frequently observed in malignant states. This may represent a mechanism by which tumor cells escape cellular immune surveillance [1-4]. Reduced or total loss of HLA class I expression on the cell surface affects the spectrum of (tumor) antigens that can be presented to cytotoxic T cells thereby limiting the anti-tumor immune response.

HLA expression is regulated by a number of mechanisms that involve gene expression, HLA complex formation, HLA complex/antigen loading and transportation to the cell surface. HLA class I cell surface expression also depends on β2 microglobulin (β2m) expression because β2m forms an essential part of the HLA class I cell surface complex.

Total or partial deletion of one chromosome 6 (HLA) or chromosome 15 (β2m) in tumors can generate HLA haplotype-loss or total-loss phenotypes, respectively. Loss of heterozygosity (LOH) of β2m may account for a substantial part of the HLA class I loss phenotypes [5-7]. The technique to study LOH of HLA and β2m has been standardized, as agreed in the HLA and Cancer component of the 13th International Histocompatibility Workshop. A selection of short tandem repeat (STR) markers minimally required for an appropriate LOH analysis and the requirements to identify LOH within a tumor have been defined, considering the existence of normal, contaminating DNA of the stroma [8]. According to Ramal et al. 2000 [8], the STR markers D15S126, D15S209 and D15S153 are minimally required to study LOH of β2m. However, in the different STR databases (Marshfield, G3, GB4, Genethron, Genemap 99) the position of these STR markers in relation to the β2m gene is not consistent. Recently, a sequence (NT_010194.8) has been published covering the genome from STR marker D15S146 to D15S117 including the β2m gene. By comparing the genome sequence with the STR databases, the positions of the STR markers relative to the β2m gene become even more controversial. This affects the interpretation of the LOH results, with respect to the region that is lost. Since the LOH of these markers has not been correlated with the expression of β2m, the relevance of these markers in relation to LOH of β2m has not been validated.

Figure 1. Map of the approximate locations of the STR markers in relation to ß2m used to determine LOH of ß2m. The location of D15S209 and D15S117 are not confirmed in the sequence (NT_010194.8).

We studied the expression of β2m in 53 HNSCC patients using immunohistochemical staining and investigated the role and usability of LOH in β2m. We also re-evaluated the availability and positions of STR markers surrounding the β2m gene region suitable for the LOH technique. Besides the STR markers minimally required for LOH analysis (D15S126, D15S209 and D15S153), we also used STR markers D15S146, D15S1028 and D15S117 (Figure 1).

3.2 Materials and Methods

The use of patient materials in this study is approved according to the guidelines of the METC protocol #96/267.

3.2.1 Immunohistochemical Staining

Immunohistochemical staining was performed on 4 μm paraffin sections of HNSCC biopsies using a three-step immuno-peroxidase technique. Visualisation of the α-β2m antibody L368 was performed using DAB [9]. The sections were analyzed and evaluated by two independent persons.

3.2.2 DNA Isolation

DNA isolation from PBL was performed using a salting-out method [10]. For DNA isolation from tumor tissue, 12 μm cryostat tissue sections were used. Each section was incubated for one hour at 51°C in 100 μl lysis mix containing 2 μl Tween 20 (Riedel de Haen, Seelze, Germany), 10 μl PCR buffer II (PE Biosystems, Foster City, CA, USA), 4 μl 25 mM $MgCl_2$ (Roche, Mannheim, Germany), 4 μl Proteinase K (10 mg/ml) and deionized water to a final volume of 100 μl. During incubation, samples were gently vortexed to enhance lysis. After one hour, the lysate was incubated at 95°C for 5 minutes to inactivate Proteinase K activity. The lysate was stored at -20°C.

3.2.3 LOH and STR Marker Analysis

LOH was performed with microsatellite markers D15S126, D15S209, D15S153, D15S146, D15S1028 and D15S117. PCR primer pairs for all microsatellite markers were obtained from the GeneDataBank (http://www.GDB.org) and are summarized in Table 1. The PCR contained 5 μl 10× buffer II (Roche), 4 μl 10 mM dNTPs (Gibco, BRL, Breda, the Netherlands), 1.5-3.5 mM $MgCl_2$ (Roche), 0.3 μl Amplitaq DNA polymerase 5 U/μl (Roche), 1 μl 3' primer (6 pmol/μl) and 1 μl 5' primer (6 pmol/μl). The D15S117 primer mix contained 10 μl 5× buffer H (Invitrogen, Breda, the Netherlands, pH 9.0, $MgCl_2$ 17.5 mM) with no additional $MgCl_2$. One μl 100 ng/μl control (PBL) DNA was added to the control PCR and 3 μl DNA from the tumor DNA was added to the tumor specific PCR. The total volume was set to 50 μl with deionized water. After mixing and addition of two drops of paraffin oil, the amplification was

performed in a PE 480 thermal cycler (PE Biosystems, Nieuwerkerk a/d IJssel, the Netherlands) for 30 cycles as follows: 94°C for 30 seconds, 62°C or 64°C for 30 seconds, 72°C for 45 seconds. Amplification was preceded by a denaturing step at 94°C for 4 minutes and completed with an extension step at 72°C for 5 minutes. Annealing temperature and optimal $MgCl_2$ concentration are shown in Table 1.

Table 1. Microsatellite markers flanking β2m on chromosome 15 suitable for LOH analysis

STR	Size [a]	Alleles	Repeat	% Het [b]	Sequence 5'primer	Sequence 3'primer	Ta (ºC)	$MgCl_2$ (mM)
D15S153	194-226	12	CA	86	AGTACCTGAAAGGGTGGG	GATCAGTGTAGGCTCCAAA	64	3,0
D15S117	132-150	9	CA	78	GCACCAACAACTTATCCCAA	CCCTAAGGGGTCTCTGAAGA	62	3,5*
D15S209	190-212	10	CA	77	AAACATAGTGCTCTGGAGGC	GGGCTAACAACAGTGTCTGC	62	2,0
D15S126	188-218	11	CA	82	GTGAGCCAAGATGGCACTAC	GCCAGCAATAATGGGAAGTT	62	1,5
D15S1028	171-187	9	CA	82	TGTCCTGAAATTCCCAAC	GAACTGTGCTCTGTGCTC	62	2,0
D15S146	217-229	7	CA	68	GGAAGCCTGACTTTATATCCG	ATGTCTGTTCAGATCCTTTGC	62	1,5

a) size range in base pairs

b) percentage heterozygosity

*) $MgCl_2$ included in PCR buffer H

PCR products were diluted 1:3 with loading buffer containing 0.5 µl ROX size standard (Applied Biosystems, Foster City, CA, USA), heat-denatured at 95°C for 2 minutes and placed on ice before electrophoresis on 6% polyacrylamide gels. An ABI PRISM 373 genetic analyser (Applied Biosystems, Foster City, CA, USA) with Genescan Analysis software 3.1 and Genotyper software was used for STR marker analysis. STR marker PCR product signals over 6,000 mean fluorescence intensity (MFI) or below 100 MFI were considered not reliable. Therefore, PCR products over 6,000 MFI were diluted 1:4 and PCR products below 100 MFI were repeated with a higher DNA concentration in the PCR reaction. LOH was calculated as described by Ramal et al. 2000 [8].

3.3 Results and Discussion

Representative illustrations of tumors with negative and positive immunohistochemical staining of tumor cells are shown in Figure 2. The results of the immunohistochemical analysis and the LOH analysis are summarized in Table 2. Out of the 53 tumors, 26 showed LOH of at least one STR marker. However, most of these tumors showed LOH for the majority of the STR markers investigated. Apparently, in most cases the size of the affected genomic region was relatively

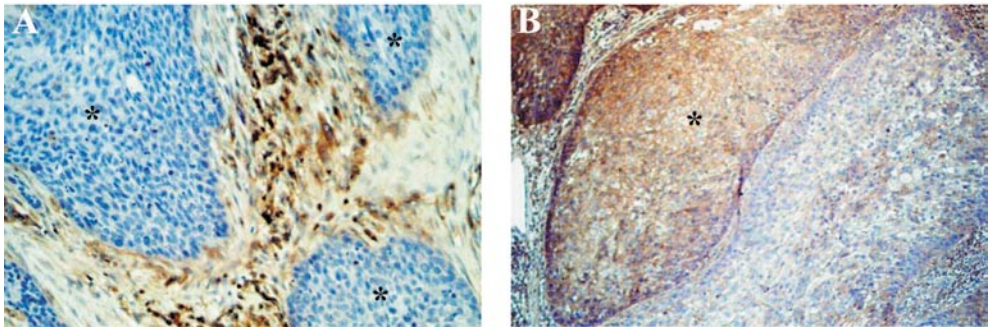

Figure 2. A) negative and B) positive immunohistochemical staining of tumor tissue (*) using α-ß2m mAb L368. The surrounding stromal tissue shows positive staining.

large, possibly larger than the region covered by these markers. It is likely that one or two STR markers close to β2m are more representative than two STR markers at a distance from the gene, although the data from the tumors we tested do not support this theory. In non-continuous STR LOH patterns, as shown in Table 2 (e.g. tumors 26 and 36), both chromosomes might be affected.

The immunohistochemical data were correlated with the LOH data. Four tumors with negative β2m staining show no LOH of the STR markers close to the β2m gene. In these cases, mechanisms other than LOH (e.g. mutations or methylation) might account for the loss of β2m expression. In the other 7 tumors showing negative β2m staining, LOH for a majority of the STR markers used, was detected. In these cases the LOH contributed to the loss of β2m expression. Since LOH of β2m leaves one allele of β2m intact, it can not solely explain this total loss of β2m expression. However, the likelihood of total loss of expression is increased because there is no "back-up" allele present. In these tumors, the remaining β2m allele is likely to have been affected (for example, by mutations or methylation) although mutations in the β2m gene are not a frequent event in HNSCC [9].

It is clear that LOH of β2m can not totally explain the cell surface expression of the gene product. Numerous other mechanisms may cause loss of expression in cases in which LOH is not present. Even so, when LOH is detected, it does not necessarily imply that β2m expression is affected. The β2m allele still present may compensate for the loss of the other allele. Moreover, when LOH is detected using STR markers and DNA is isolated from total tissue sections, loss of heterozygosity can not be distinguished from amplification. Due to the contaminating stromal tissue DNA, the signal reduction of a STR marker allele is not reduced by 100%. Since LOH is calculated as the ratio of both STR alleles in the tumor tissue compared to the ratio in normal tissue (PBL), no distinction can be made between the loss of one allele versus the amplification of the other allele. We found four tumors with LOH of the majority of the STR markers, but *overexpression* of β2m was identified

Table 2. LOH data of six STR markers on chromosome 15q correlated with immunohistochemical α-β2m staining

			LOH Chromosome 15q [c]					
Tumor	% Tumor Tissue [a]	α-ß2m IH [b]	S153	S117	S209	S126	S1028	S146
1	30	h-	○	●	NI	●	NI	●
2	nd	h	○	○	○	○	NI	NI
3	nd	++	○	○	○	○	NI	○
4	50	++	○	○	○	○	○	NI
5	20	+	●	●	●	●	●	●
6	20	+	○	○	○	○	○	○
7	80	h+	○	○	○	●	NI	NI
8	80	+	○	○	●	●	○	NI
9	70	+	○	●	○	●	●	NI
10	80	+	○	NI	○	○	○	NI
11	50	h	NI	○	○	○	○	○
12	30	h-	●	●	●	●	NI	●
13	60	h+	○	○	○	○	NI	○
14	30	-	○	○	○	NI	○	NI
15	70	++	●	●	●	●	NI	●
16	70	-	●	○	○	NI	○	○
17	20	+	NI	●	●	●	NI	●
18	60	h	○	○	○	NI	○	○
19	40	h+	○	○	○	○	○	○
20	70	h	○	○	○	○	●	○
21	70	-	○	○	○	NI	○	○
22	50	-	NI	●	NI	NI	●	○
23	30	++	NI	○	○	○	○	○
24	40	++	●	○	●	NI	●	NI
25	50	-	NI	●	●	●	NI	NI
26	80	h -	○	●	○	●	●	○
27	70	h+	○	NI	○	○	○	NI

a) percentage of tumor tissue in the tissue section (nd not determined)

b) immunohistochemical staining results performed with antibody α-β2m, (++) overexpression, (+) positive expression, (h+) positive expression with few small negative fields, (h) heterogeneous expression, (h-) negative expression with few small positive fields and (-) negative expression

c) LOH data, ● LOH of the marker, ○ no LOH of the marker and (NI) STR marker is not informative due to homozygosity of the sample

Table 2. LOH data of six STR markers on chromosome 15q correlated with immunohistochemical α-β2m staining

			LOH Chromosome 15q [c]					
Tumor	% Tumor Tissue [a]	α-ß2m IH [b]	S153	S117	S209	S126	S1028	S146
28	50	+	○	○	○	○	○	○
29	50	-	NI	●	○	●	●	NI
30	20	++	○	○	○	○	○	NI
31	50	+	NI	○	NI	○	○	○
32	0	+	○	○	○	NI	NI	NI
33	40	+	○	○	○	○	○	○
34	<10	+	○	NI	○	NI	○	○
35	70	+	○	○	○	○	NI	○
36	70	+	○	●	●	○	●	●
37	50	+	○	○	○	○	○	○
38	60	+	○	○	○	●	○	○
39	70	+	○	○	○	○	○	○
40	50	h-	●	●	●	●	●	●
41	60	++	○	○	○	○	●	○
42	10	++	●	●	●	●	●	●
43	80	++	NI	●	●	NI	●	○
44	40	h+	○	NI	●	●	○	NI
45	60	+	○	○	○	○	○	○
46	50	+	○	○	●	○	NI	○
47	30	h+	NI	●	●	●	●	●
48	50	+	○	○	NI	○	NI	NI
49	70	++	○	NI	NI	○	○	○
50	10	-	○	○	NI	NI	○	○
51	60	+	○	○	○	○	○	○
52	<5	+	●	NI	●	●	●	●
53	60	+	●	○	NI	●	●	●

a) percentage of tumor tissue in the tissue section (nd not determined)

b) immunohistochemical staining results performed with antibody α-β2m, (++) overexpression, (+) positive expression, (h+) positive expression with few small negative fields, (h) heterogeneous expression, (h-) negative expression with few small positive fields and (-) negative expression

c) LOH data, ● LOH of the marker, ○ no LOH of the marker and (NI) STR marker is not informative due to homozygosity of the sample

by immunohistochemical staining. It is likely that amplification of the β2m gene region occurred rather than loss of heterozygosity in these cases. In an additional eight tumors, LOH of at least two STR markers was detected although the β2m expression was positive. There are two possible explanations for this. First, these tumors have LOH while the "backup" allele compensates for the lost allele. Second, there is amplification of one allele while the expression is kept at a "normal" level.

Although the use of microsatellite (STR) markers for studying LOH is a powerful technique due to the high degree of polymorphism of these markers and their distribution throughout the genome, they also have a number of disadvantages. The location of these markers is not as evident as described in different databases. Moreover, it is very important to be able to distinguish allele amplification from allele loss. Without additional techniques, STR marker analysis is not conclusive for LOH of β2m or loss of a genomic region in general.

Using microdissection to isolate the tumor tissue from the tissue section has a number of advantages. First, tissue sections containing less than 60% tumor tissue can be studied because the contaminating stromal tissue DNA is excluded. Second, in case a STR allele is lost, the signal reduction of the lost allele should be 100%. We applied microdissection on a number of tumors with less than 60% tumor tissue. The majority of the tumors did not show 100% signal reduction but only a minor improvement of signal reduction (data not shown). Heterogeneity of the isolated tumor tissue could explain this phenomenon. However, HNSCC are considered homogeneous tumors. The finding that after microdissection of the tumor tissue not always 100% loss of a STR allele is obtained, may be explained by amplification of (a part of) the chromosome. LOH does not solely represent loss of one allele: both alleles can still be present in the tumor but in a disproportional ratio. Even microdissection can not distinguish this difference. As mentioned before, the exact location of the β2m gene in relation to the surrounding STR markers is unknown. This makes it hard to select two (flanking) STR markers which are most representative for β2m gene LOH. Above all, loss of heterozygosity is quite different from amplification. Fluorescence *in situ* hybridization (FISH) using a β2m specific probe may solve these problems. One can visualise the presence or absence of a genomic DNA region of minimally 20-30 kbp. Since a large genomic region flanking β2m is affected, a centromeric probe for chromosome 15 might also be sufficient. Gene- or Fiber-FISH will be necessary if one wants to characterize specific β2m gene loss.

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Chapter 4

High Level of Chromosome 15 Aneuploidy in Head and Neck Squamous Cell Carcinoma Lesions Identified by FISH Analysis: Limited Value of β2 Microglobulin LOH Analysis.

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Abstract: In cancer research, loss of heterozygosity (LOH), defined by microsatellite markers, is frequently used in the identification of gene loss. Especially genomic alterations in the human leukocyte antigen (HLA) genes and the β2 microglobulin (β2m) gene on chromosome 15 are of interest regarding their function in the immune system. Because LOH analysis detects any allelic imbalance and not just allelic loss, we evaluated the LOH analysis in 11 Head and Neck Squamous Cell Carcinoma (HNSCC) lesions using fluorescence *in situ* hybridization (FISH). The 11 tumors were selected out of 53 HNSCC lesions based upon β2m LOH analysis and β2m expression. Centromere 1 and 15 FISH were developed to determine the chromosome 15 copy number. Sequence-based mutation analysis of β2m was conducted on tumors without β2m expression; no mutations in the coding sequences were found. For five HNSCC lesions with LOH and β2m expression, centromere 15 FISH indicated gain rather than loss. In the majority of the 11 HNSCC lesions, FISH showed centromere 1 and 15 heterogeneity throughout the tumor. Moreover, FISH indicated a more complex chromosome 1 and 15 distribution than could be concluded from microsatellite LOH analysis. Our results show that microsatellite LOH analysis does not represent the β2m gene copy number and support the results obtained from comparative genomic hybridization (CGH) studies. Conclusions on genomic alterations in tumors can not be based on LOH data only, but depend on the results of immunohistochemical staining, FISH and CGH.

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4.1 Introduction

The Human Leukocyte Antigens (HLA) cluster on the short arm of chromosome 6 and play a central role in the immune system. On the surface of human cells, HLA molecules present antigenic peptides to the immune system [1-3]. HLA class I antigen presented peptides reflect the intracellular events. This way, infected cells can be distinguished from healthy cells by the immune system and an adequate immune reaction can be invoked. Downregulation of HLA expression is frequently observed in malignancies [4]. Therefore, HLA molecules are considered to be involved in tumorigenesis [5-8]. Mutations in the HLA genes and, in particular, the β2m gene can alter the HLA repertoire on the cell surface significantly [9-11]. For example, loss of β2m expression results in total loss of HLA class I antigen expression, because β2m is essential for HLA class I antigen complex stability [12]. Other phenomena in tumors, like gene methylation and genomic loss, also change the expression of these genes. Tumor cells benefit from abnormalities in HLA expression [6]. Reduced HLA class I antigen expression on the tumor cell surface affects the number and variety of tumor-associated antigens (TAA) that can be presented to cytotoxic T lymphocytes (CTL) [13]. This alters the possibilities of an effective CTL mediated immune surveillance and elimination of the tumor cell. Loss of HLA class I antigen cell surface expression shifts the immune surveillance from CTL to natural killer (NK) cells which eliminate cells that escape a CTL response. Instead of losing complete HLA class I antigen expression, tumor cells may have found a balance between HLA class I antigen expression and loss. One could speak of an HLA expression "escape-window" to evade both NK cell and CTL immune responses. Hence, it is not surprising that the majority of tumors show a wide variety of different loss phenotypes (e.g. haplotype-loss, locus-loss and allelic-loss phenotypes as described by Garrido et al. 1997) instead of total loss [8].

To define the different HLA loss phenotypes and understand the role of HLA in tumor development, expression of HLA molecules in tumors is characterized using HLA locus specific anti-sera. However, this technique has some limitations since the most frequently occurring phenotypes (e.g. haplotype-loss and allelic-loss phenotypes) are difficult to characterize due to the lack of sufficient HLA allele specific anti-sera. It is currently accepted that genomic loss in the HLA region (e.g. loci and alleles) and chromosome 15 (β2m) is a common cause of all types of HLA-loss phenotypes [8, 14-17]. Jordanova *et al.* 2003 demonstrated that multiple defects in the β2m locus, such as mutations and deletions, caused β2m expression loss and impaired HLA class I antigen expression [18]. Loss of a genomic region is often studied using short tandem repeat markers to characterize loss of heterozygosity (LOH) patterns in tumors. Because β2m is required for HLA cell surface expression, we correlated LOH data of a region of chromosome 15, including the β2m gene, with immunohistochemical staining of β2m in head and neck squamous cell carcinoma

(HNSCC) lesions [17]. Tumors with negative β2m immunohistochemical staining showed "LOH" as well as "no LOH". Because LOH reflects the loss of one of the alleles and therefore can not cause expression loss, we performed sequencing based mutation analysis (SBMA) to study whether additional mutations in the β2m protein coding sequence were responsible for the β2m expression loss. However, we did not find any mutations in the β2m protein coding sequence.

Of the β2m immunohistochemical positive tumors, the majority showed no LOH, but 5 HNSCC lesions showed LOH while β2m was normally or overexpressed [17]. Tumors showing LOH are considered to have affected gene and protein expression because there is only one copy of the genomic region or gene present. These five tumors do not fit this theory. Microsatellite marker LOH analysis can not distinguish loss from gain; any allelic imbalance gives a LOH ratio not equal to one. Besides, proportional chromosome copy numbers (e.g. 2, 4, etc.) can not be discriminated. Therefore, it could well be that these tumors have three β2m alleles instead of one β2m allele. We therefore studied the chromosome 15 copy number in these HNSCC lesions using FISH with a centromeric probe for chromosome 15 and included chromosome 1 as control. Chromosome 15 fluorescence *in situ* hybridization (FISH) reflects the β2m gene copy number. Only in tumors with β2m gene-specific loss chromosome 15 FISH will underestimate the β2m gene loss.

4.2 Materials and Methods

4.2.1 Tissue Samples

The 53 HNSCC lesion specimens used in this study were obtained surgically at our department between 1996 and 2001 and underwent standard tissue processing. β2m immunohistochemical staining and microsatellite LOH studies were performed on all tumors. Seven HNSCC lesions with a negative β2m immunohistochemical expression pattern were studied for mutations using SBMA. Based on the immunohistochemical staining and the LOH data, 11 (three mouth-cavity carcinomas, six oropharynx tumors and two hypopharynx carcinomas) of the 53 HNSCC lesions were selected for FISH to validate the β2m LOH data. These included four HNSCC lesions with a positive β2m expression and LOH, four with a heterozygous β2m expression and LOH and three HNSCC lesions with a negative β2m expression and no LOH.

4.2.2 Immunohistochemical Staining

Immunohistochemical staining was performed on 4-µm thick paraffin sections of HNSCC lesions. Sections were mounted on silan-coated glass slides and baked overnight at 60ºC. Heat-induced epitope retrieval in 10 mM tri-sodium-citrate-2-hydrate buffer pH 6.0 was necessary, prior to immunohistochemical staining. The anti-β2m monoclonal antibody (mAb) L368 was diluted 1:200 in phosphate-buffered

saline (PBS) containing 1% bovine serum albumin (BSA) and 0.2% sodium azide. For immunohistochemical staining, the three-step biotin-streptavidin-peroxidase technique was used with 3,3'-diaminobenzidine.4HCl (DAB) as chromogen. Sections were analyzed by two independent observers. Discrepancies were re-evaluated in the presence of a third independent observer.

4.2.3 Loss of Heterozygosity

LOH studies were carried out as described previously [17]. In short, DNA was isolated from peripheral blood lymphocytes (PBL) and HNSCC lesion cryostat tissue sections. To detect LOH of the β2m gene, we used an extended panel of microsatellite markers (D15S126, D15S209, D15S153, D15S146, D15S1028 and D15S117) compared to the panel described in literature [19]. Genescan signals over 6,000 Mean Fluorescence Intensity (MFI) or below 100 MFI are considered to be not reliable. LOH was calculated as described by Ramal, et al. 2000 [19].

4.2.4 Microdissection

Laser-catapulted microdissection (LCM) was performed on frozen HNSCC lesion sections containing less than 60% tumor and on HNSCC lesions showing approximately 25% LOH [20]. Tissue sections were mounted on Pennfoil-coated glass slides, air-dried for 30 min, stained with methylene blue (0.1% in ethanol) for 10 s and rinsed with water. After 30 min of drying, small tumor fields of approximately 500 cells were microdissected with a PALM microdissection microscope (PALM, Germany). Subsequently, DNA was released by incubating the microdissection tissue field for one hour at 51ºC in a lysis mix containing 2 µl Tween 20 (Riedel de Haen, Germany), 10 µl PCR Buffer II (PE Biosystems, USA), 1 mM $MgCl_2$ (Roche, Germany), 4 µl Proteïnase K 10 mg/ml (Roche, Germany) and deionized water to a final volume of 100 µl. During incubation, samples were gently vortexed to enhance lysis. After one hour, the lysate was incubated at 95ºC for 5 min to inactivate Proteïnase K. Finally, the DNA was stored at -20ºC.

4.2.5 Sequence-Based Mutation Analysis

β2m exons 1, 2 and 3 were amplified using intron sequence-specific primers with an universal M13 template sequence as summarized in Table 1. Each PCR reaction contained 10 µl 5× PCR buffer C (Invitrogen, The Netherlands), 5 µl dNTP's 10 mM (Invitrogen, The Netherlands), 0.2 µl 5 U/µl Taq Polymerase (Roche, USA), 0.2 µl forward primer (50 pmol/µl), 0.2 µl reverse primer (50 pmol/µl), 5 µl DNA and deionized water to a final volume of 50 µl. Amplification was performed in a Perkin Elmer 9600 (Applied Biosystems, the Netherlands) using the following program: a preheating step at 94°C for 3 min, 30 cycles of 96°C for 20 s, 58°C for 30 s, 72°C for 45 s and finally a hold at 4°C. PCR products were used in a re-PCR before

Table 1. β2 microglobulin SBMA primers

β2m Exon	5' Primer Sequence 3'
5' Exon 1	*tgtaaaacgacggccagt*GTCCTGCGGGCCTTGTCCTGATT
3' Exon 1	*caggaaacagctatgacc*CGCACCCCCTTCCCCACTCC
5' Exon 2	*tgtaaaacgacggccagt*ACCAAGTTAGCCCCAAGTGA
3' Exon 2	*caggaaacagctatgacc*CAATCCCAATATGCAGAGTGTT
5' Exon 3	*tgtaaaacgacggccagt*CCTATTCTGCCAGCTTATTTCT
3' Exon 3	*caggaaacagctatgacc*ACCTTCTTCATGCCACTCACA

β2 microglobulin intron sequence specific PCR primers containing an universal M13 template sequence depicted in *italics.*

sequencing. The re-PCR is identical to the first PCR except for the 5 µl DNA; this is exchanged for 5 µl PCR product. Re-PCR products were loaded on an 2% agarose gel and cut out of the gel after electrophoresis. The PCR products were isolated from the gel by centrifugation over glass wool at 20,800 ×g for 25 min. Precipitation was performed by adding 10 µl 3 M sodium acetate pH 5.2, 200 µl ethanol pro-analysis and incubating at –20°C for 30 min. Precipitation was continued by centrifugation at 20,800 ×g for 30 min. Subsequently, the DNA pellet was washed with 400 µl 70% ethanol, centrifuged at 20,800 ×g for 5 min and dissolved in 25 µl deionized water, storing it at 4°C. Sequencing was done using the Big Dye Terminator Kit (PE Applied Biosystems, USA). Eight µl of Big Dye Terminator mix, 0.64 µl M13 forward or reverse primer (5 pmol/µl) and 6.36 µl deionized water were added to 5 µl purified PCR product. The sequence reaction was conducted in an Amplitron® II Thermolyne (Beun de Ronde, the Netherlands) using the following program: 10 s at 96°C, 10 s at 50°C and 2 min at 60°C for 20 cycles followed by a final step of 96°C for 10 s. Sequence products were precipitated and prepared for loading on a ABI 377 Sequencer Gel as described in the ABI 377 Sequencer manual. Sequence data were analyzed using ABI's Sequence Analysis Software and the multisequence analysis software Polall [21]. SBMA analysis was performed in duplo to exclude any inaccuracies due to the use of Taq polymerase in a re-PCR.

4.2.6 *Fluorescence in situ* Hybridization

Six-micron thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on silan-coated glass slides. The sections were baked overnight by incubating at 58°C, deparaffinized in xylene, rehydrated in an ethanol series and air-dried. Of each tumor, one section was stained with haematoxylin-eosin (HE) for morphologic review. For FISH, sections were pepsinized for 10 min at 37°C in 500 U/ml pepsin in 0.1 M glycin/HCl pH 2.0, washed, and autoclaved in PBS. After the slides reached room temperature, they were dehydrated in an ethanol series and air-dried.

Digoxigenin labeling of the centromere 1 and 15 probes (ATCC, Rockville Maryland, USA) was performed using a nick translation kit (Roche, USA) according to the manufacturers manual. The nicked digoxigenin-labeled probes were checked for size (ideally 100 bp - 600 bp) on an 1.5% agarose gel and precipitated using 1 volume sonicated haring sperm DNA (HSD), 0.1 volume 3 M sodium acetate pH 5.2 and isopropanol. The probe was dissolved in 1 mM Tris 0.1 mM EDTA to a final concentration of 20 ng/µl. The probe hybridization mixture (200 ng probe in 40 µl hybridization buffer containing 50% formamide, 2× SSC, 10% dextran sulfate) was boiled for 5 min and subsequently cooled on ice for 5 min. After pipetting the hybridization mix onto each tissue section and applying coverslips, codenaturation of target and probe DNA was achieved by placing the slides on a hot plate for 10 min at 98°C. The slides were incubated overnight in a moisture environment at 37°C and subsequently washed (50% formamide, 2× SSC, 0.05% Tween 20) for 5 min at room temperature and 10 min at 45°C. Stringency wash was followed by washing in 4× SSC/ 0.05% Tween 20 and blocking with 100 µl block buffer (5% non-fat milk in 4× SSC/ 0.05% Tween 20) for 10 min. Probe hybridization was detected by incubation with 100 µl FITC-labeled α-digoxigenin (diluted 1:10 in block buffer) for 30 min followed by washing (0.05% Tween 20/PBS). Nuclei were stained using 100 µl propidium iodide (1:10,000 in PBS) for 20 s. Finally, the sections were washed twice in 0.05% Tween 20 in PBS and covered with Vectashield (Vector Laboratories Inc., CA, USA) and a coverslip. For evaluation, pictures were taken from two different tumor regions in the slide using a Confocal Laser Scan Microscope (CLSM, MRC-1000, Bio-Rad Microscience Ltd, UK). The number of fluorescent signals per cell was categorized into five groups: no signal, one signal, two signals, three signals and more than three fluorescent signals. On average, 250 cells were counted per photo with an absolute minimum of 150 cells. Each picture was evaluated by two independent persons. Overlapping nuclei were not included in the analysis. Concordance of the FISH signal distribution of the two observers was tested using a Chi-square test ($p<0.05$); significant differences were re-evaluated. Hybridization efficiency was tested on two control tissues (tonsil) and PBL spots.

4.3 Results

4.3.1 Immunohistochemical Staining & Loss of Heterozygosity

Immunohistochemical staining for β2m using anti-β2m mAb L368 was performed on 53 HNSCC lesions [17]. Classification of the tumor tissue was divided into positive, negative or heterogeneous staining of which examples are shown in Figure 1. The β2m immunohistochemical staining results of the 11 tumors selected for FISH, together with the results of the β2m mutation analysis, β2m LOH data and FISH analysis, are summarized in Table 2. Detailed LOH data for all individual microsatellite markers tested on the tumors are shown in Table 3. LOH was ascribed to the tumor when microsatellite markers showed a decrease of at least 25% of one allele relative to the second in the tumor, compared to the ratio in peripheral blood lymphocytes (PBL). Complex loss patterns in cases HN36, HN26 and HN53 can arise due to loss of microsatellite markers in both chromosomes or due to heterogeneity within a tumor. Microdissection was applied when the tumor percentage in the tissue section was below 60% or when the LOH percentage was around the critical value of 25% [19]. In most cases, microdissection improved the sensitivity of the LOH detection. However, a loss percentage of 100% was rarely found. The final LOH results are summarized in Table 2[c] indicating "+" for LOH and "-" for no LOH.

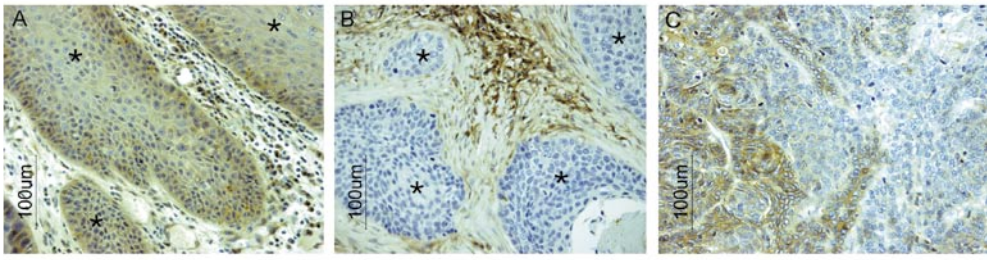

Figure 1. Peroxidase α-β2m mAb L368 immuno-stained paraffin sections of HNSCC lesions with a) positive staining; b) negative staining and c) heterogeneous staining of the tumor cells (*).

4.3.2 β2m Mutation Analysis

Of the 53 studied HNSCC lesions, seven tumors with a negative β2m immunohistochemical staining pattern were studied for mutations in the three coding exons and flanking introns of the β2m gene using SBMA. Sequences were analyzed with the multisequence analysis software PollAll, developed at our department [21]. None of the tumors showed any mutation in the exons, including the mutation hotspot in exon 1 as described for colon carcinoma and melanoma [22] (Table 2[a]).

Table 2. Overview of the 11 HNSCC lesions selected for FISH analysis

				FISH centromere 15		
Tumor	SBMA β2m [a]	IHC β2m [b]	LOH STR [c]	Field	FISH 15 [d]	Loss/Gain [e]
HN36	n.s.	+	+	A B	++ -	G N
HN01	n.s.	h	+	A B	+ +	G G
HN26	n.s.	h	+	A B	- +	N L
HN16	-	-	-	A B	+ +	L L
HN40	n.s.	h	+	A B	+ ++	L/G L
HN12	n.s.	h	+	A B	+ +	L L
HN42	n.s.	++	+	A B	+ +	L G
HN17	n.s.	+	+	A B	+ +/-	L L/G
HN14	-	-	-	A B	+/- +/-	L L
HN50	-	-	-	A B	+ ++	G G
HN53	n.s.	+	+	A B	+/- +/-	L/G L/G

a) sequence based mutation analysis (SBMA) of the three coding exons of the β2m gene, no mutations (-), not selected for mutation analysis (n.s.)

b) immunohistochemical β2m staining, strong positive(++), positive (+), negative (–) and heterogeneous (h) staining

c) loss of heterozygosity, LOH (+), no LOH (-)

d) FISH centromere 15, statistical difference $p<0.05$ (++), clear distribution difference (+), less clear distribution difference (+/-) and no distribution difference (-)

e) normal (N), loss (L), gain (G) and loss or gain (L/G) of centromere 15 FISH signal in tumor cells

4.3.3 Fluorescence in situ Hybridization

FISH was performed on all tumor samples summarized in Table 2. As a control for chromosome 15 FISH signals in paraffin-embedded tissue sections, centromere 1 FISH was performed on the same HNSCC lesion sections. Hybridization efficiency was tested on PBL spots and two control (tonsil) tissues. Of each HNSCC lesion slide, two photos were taken each for centromere 1 and centromere 15 FISH of different tumor tissue fields within a tumor (field A and field B). An example of a tumor with amplification and a tumor showing loss of chromosome 15 is depicted in Figures 2A and 2B, respectively. Each field was analyzed separately. The FISH results are included in Table 2[d & e] for centromere 15 FISH.

Centromere 1 and 15 FISH show approximately the same frequency distribution of the FISH signals (Figure 3). Obviously, both centromere 1 and 15 FISH distribution

Table 3. Microsatellite LOH on chromosome 15q

Tumor	D15S153	D15S117	D15S209	D15S126	D15S1028	D15S146
HN36	○	●	●	○	●	●
HN01	○	●	NI	●	NI	●
HN26	○	●	○	●	●	○
HN16	●	○	○	NI	○	○
HN40	●	●	●	●	●	●
HN12	●	●	●	●	NI	●
HN42	●	●	●	●	●	●
HN17	NI	●	●	●	NI	●
HN14	○	○	○	NI	○	NI
HN50	○	○	NI	NI	○	○
HN53	●	○	NI	●	●	●

Loss of heterozygosity results of the 6 individual microsatellite markers surrounding the β2m gene on the long arm of chromosome 15. Non-informative marker (NI) due to homozygosity, ● loss of heterozygosity, ○ no loss of heterozygosity.

are heterogeneous within the majority of the tumors and show loss as well as amplification. Therefore, it can be concluded that chromosome 1 varies at least as much as chromosome 15. Apparently, both chromosomes are affected in HNSCC lesions. For centromere 1 and 15 FISH respectively, four (HN16, HN26, HN36 and HN01) and three (HN40, HN36 and HN50) tumors have a frequency value outside the normal distribution ($p<0.05$). These tumors are statistically different, indicated by "++" in Table 2[d]. In Figures 3A and 3B, tumor numbers are also included.

An overview of the centromere 15 FISH signal frequency of each tumor is given cumulatively in Figure 4. In a normal tissue section, the majority of the cells have 2 FISH signals. Due to cutting the tissue sections, a certain percentage of normal cells with two chromosome 15 copies have truncated nuclei and show one or zero FISH signals per nucleus instead of two. Moreover, proliferating or aneuploid tumor cells with four or more chromosome copies may show zero, one, two, three or more FISH signals as a result of truncated nuclei. Although the use of isolated nuclei would circumvent this problem, the tissue morphology is lost. This makes it very difficult to distinguish the nuclei of tumor cells from those of other cells. Our intended control, chromosome 1 FISH, varies comparable to the chromosome 15 results (Figure 3) and apparently is not suitable. However, the tonsil control tissue is a good control for the normal chromosomal FISH distribution in paraffin-tissue sections. The mean distribution of the control tissues is depicted in Figure 4 as "control".

To discriminate loss from gain, we analyzed the centromere 15 signal distribution of each tumor and assigned loss when at least 40% of the cells show 0 and 1 FISH

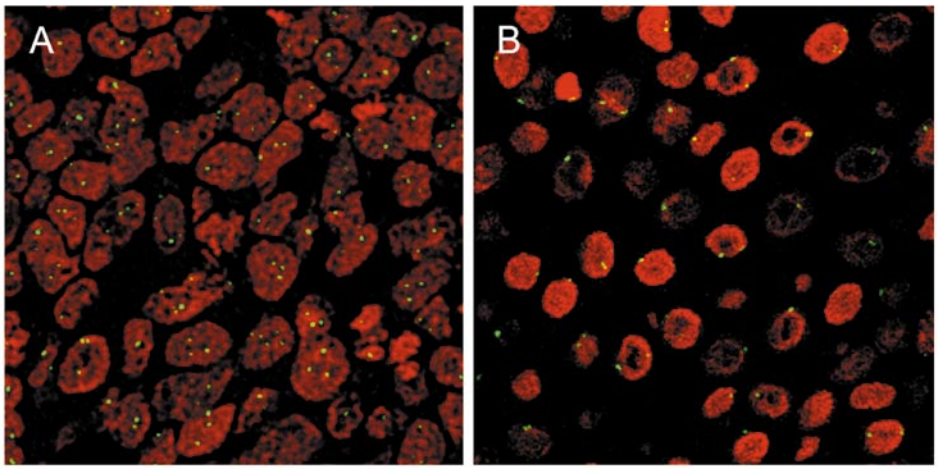

Figure 2. Centromere 15 FISH of HNSCC lesions showing a) gain and b) loss of chromosome 15. Nuclei were stained red using propidium iodide while the centromere 15 probe was visualized in green using FITC-labeled α-digoxigenin (1:10,000). Overlapping nuclei were not included in the analysis.

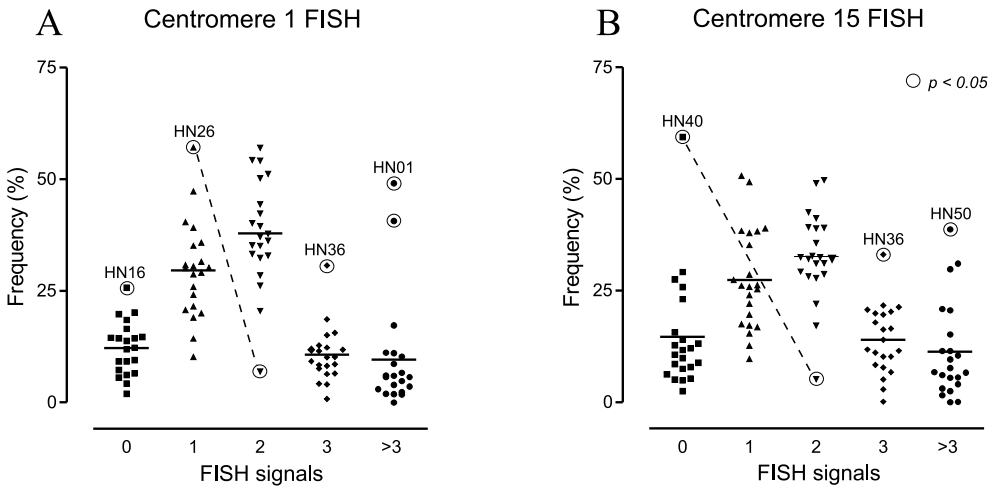

Figure 3. Frequency distribution of centromere 1 (A) and centromere 15 (B) FISH for both tumor fields of the 11 HNSCC lesions. The median per FISH signal is indicated by a horizontal line. FISH signal frequencies of tumor fields with $p<0.05$ are indicated by a circle and connected by a dotted line when the frequencies belong to one tumor field.

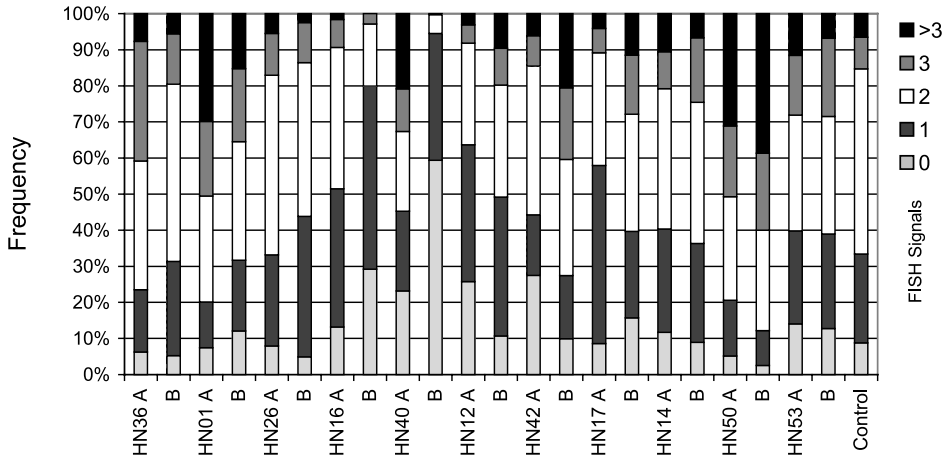

Figure 4. Cumulative presentation of centromere 15 FISH frequencies of tumor field A and B of 11 HNSCC lesions studied with centromere 1 and 15 FISH. The mean of the control tonsil tissues is depicted as "control". HN36 B and HN26 A are examples of a "normal" FISH distribution while HN16 B and HN12 clearly show loss of centromere 15. HN36 A and HN50 are examples of HNSCC lesions with gain of chromosome 15.

signals. Gain was assigned when at least 30% of the cells show 3 and more than 3 FISH signals. These criteria were based upon the FISH signal distribution of the control tissues. Tumors with distributions that are clearly, but not significantly different, like HN01, HN16, HN12, HN50 A, are indicated by a "+" in Table 2[d]. HNSCC lesions HN17 B, HN14 and HN53 are borderline cases: it is difficult to assign loss or gain (Figure 4). This is indicated by "+/-" in Table 2[d]. Although unlikely, both loss and gain may be occurring in HN17 B and HN53. Normal distributions like HN36 B and HN26 A are indicated by "–" in table 2[d]. Normal (N) chromosome 15 distribution, loss (L), gain (G) and "loss or gain" (L/G) of chromosome 15 is indicated per tumor field in Table 2[e].

4.3.4 Four Tumors with LOH and β2m Expression in Detail

The centromere 15 FISH results for tumor ***HN36*** field A and B differ the most in the "three FISH signals" category (Figure 5A). Tumor field A has significant ($p<0.05$) more cells with three FISH signals per nuclei than all other tumors (Figure 3). Field B has a normal distribution of FISH signals. Within this tumor, a heterogeneous chromosome 15 distribution is observed. In contrast to LOH, as indicated by microsatellite marker analysis (Table 2[e]), this tumor rather has gain than loss of chromosome 15. This explains the positive expression of β2m. Moreover,

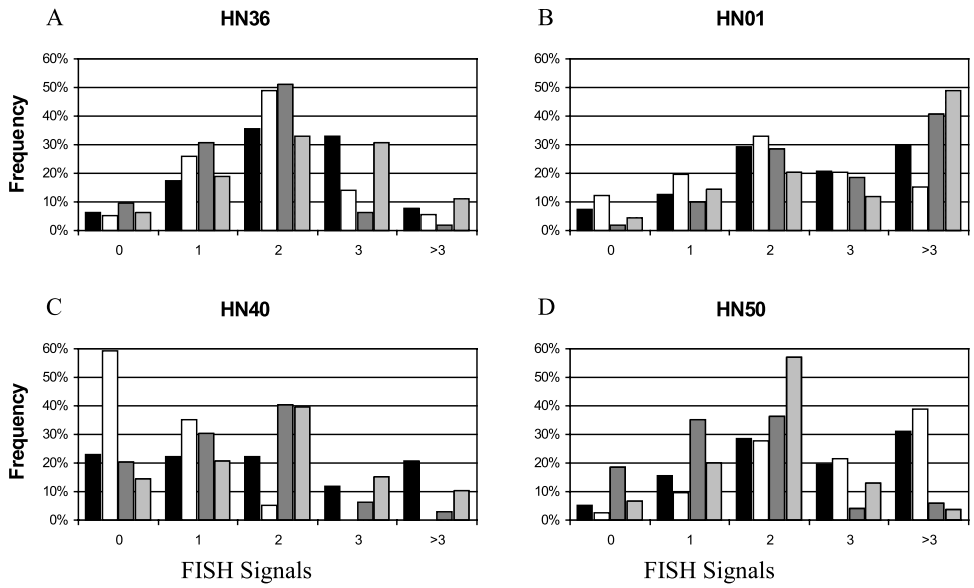

Figure 5. Centromere 15 and 1 FISH frequency distribution of four different HNSCC lesions. The number of cells with 0, 1, 2, 3, >3 FISH signals are depicted as the percentage of the total of cells counted. An absolute minimum of 150 cells per tumor field was counted. FISH centromere 15 tumor field A ■ and tumor field B □ ; centromere 1 tumor field A ▩ and tumor field B ▩ . In all four HNSCC lesions, the heterogeneity between the tumor fields for centromere 15 as well as 1 is clearly visible.

centromere 1 FISH is heterogeneous and even shows gain of chromosome 1 in tumor field B (Figure 5A). It is tempting to assume that centromere 15 tumor field A corresponds with centromere 1 tumor field B and vice versa.

The centromere 15 FISH distributions of the two tumor fields of tumor ***HN01*** differ the most in the "more than three FISH signals" category (Figure 5B). Both tumor fields show gain of chromosome 15 (Figure 4), whereas LOH was concluded based upon the microsatellite marker analysis. Centromere 1 FISH shows gain for both tumor fields, indicating that chromosome 1 is also affected in this tumor. The immunohistochemical β2m staining shows heterogeneity (Table 2[b]).

In contrast to the majority of the tumors, tumor ***HN40*** field A shows a completely different distribution of centromere 15 FISH signals (Figure 4). With the exception of the more than three FISH signals category, there is an equal distribution of FISH signals in this tumor field (Figure 5C). Field B has a large percentage of cells with 0 and 1 centromere 15 signal. Loss of one chromosome 15 is confirmed with the microsatellite markers although loss of both chromosomes can not be detected. Immunohistochemical staining shows a heterogeneous β2m expression pattern,

which is not surprising regarding the heterogeneity of the centromere 15 distribution. Both fields of the centromere 1 FISH are heterogeneous and show loss.

Tumor ***HN50*** has a negative β2m expression, no mutations in the β2m exons and shows no LOH (Table 2). FISH indicates gain of chromosome 15 in both tumor fields (Figure 5D). Because the amplification arises mainly from the "more than three FISH signals per nucleus" category, the microsatellites will not detect a major non-proportional chromosome 15 copy number. This explains why the microsatellites do not detect LOH. Although centromere 15 FISH distribution of field A and B are almost identical, centromere 1 FISH distribution is heterogeneous. Field A shows loss while field B shows a normal distribution.

4.3.5 Succinct Overview of the 7 other Tumors

Tumor field A of ***HN26*** has a normal FISH distribution. Field B has approximately the same amount of cells with one chromosome 15 as with two chromosomes (Figure 4). Loss of one chromosome 15 is confirmed by the microsatellite markers. The heterogeneity of the chromosome 15 distribution is reflected in the heterogeneous β2m protein expression.

Although tumor ***HN16*** has no mutations in the exons of the β2m gene and no LOH, it shows a negative immunohistochemical β2m staining (Table 2[b]). For both tumor fields the centromere 15 FISH results indicate loss (Figure 4). In this case the microsatellite analysis does not detect the loss of chromosome 15.

Both tumor fields of ***HN12*** show approximately the same centromere 15 distribution. Only the frequencies of cells with no centromere 15 differ between the two tumor fields. The loss of chromosome 15 corresponds with the LOH data of the microsatellite markers. β2m protein expression shows a heterogeneous pattern (Table 2[b]).

Tumor ***HN42*** has some loss in field A and some gain in field B while LOH is detected by microsatellite marker analysis. In this case, the loss in tumor field A and gain in field B both contribute to the LOH percentage. Immunohistochemical staining indicates overexpression of β2m. Considering the heterogeneity of the tumor, the majority of the tumor cells may have gain of chromosome 15 which explains the overexpression of β2m.

Tumor field A of ***HN17*** has loss of one chromosome 15 while field B is on the border of loss and gain (Figure 4). Although the majority of the tumor cells show loss, immunohistochemical staining of β2m is positive.

HN53 and ***HN14*** show approximately the same centromere 15 distribution. While HN14 has no mutations in the exons of β2m, no LOH and a negative β2m protein expression, HN50 shows LOH and positive β2m expression.

From these results it can be concluded that: i) each tumor in this panel shows unique characteristics; ii) chromosome 1 as well as chromosome 15 is affected; iii) the

majority of the tumors are heterogeneous for chromosome 1 and chromosome 15 copy number within the tumor; iv) tumors clearly show a more complex chromosome 15 and 1 distribution than just loss of one (part of the) chromosome as indicated by microsatellite LOH analysis; v) based upon the microsatellite LOH results, three out eleven tumors (HN36, HN01 and HN42) are considered to have loss, whereas FISH analysis does not show any loss, but only gain; vi) heterogeneity within the tumor (field A & B) gives rise to complex expression patterns and LOH.

4.4 Discussion

In this study, 11 out of 53 tumors were selected, based on the β2m immunohistochemical staining and LOH data, for additional analysis with chromosome 1 and 15 FISH. These 11 tumors indicate the diversity of aneuploidy that occurs frequently in HNSCC lesions. Four tumors with LOH and positive β2m immunohistochemical staining were included to study whether these HNSCC lesions have gain rather than loss of chromosome 15.

Of all HNSCC lesions with a negative β2m immunohistochemical staining, none of them show a mutation in the three coding exons and flanking intron sequence of the β2m gene. It confirms that mutations in the β2m gene is not a frequent event in HNSCC lesions [23]. This is remarkable, because in colon carcinoma, melanoma and even in mice, a mutational hotspot has been found [22, 24]. Because these HNSCC lesions lack any mutations in the exons, LOH can not explain the total loss of β2m expression because one intact β2m gene is still present. However, mutations in the promoter or methylation of the β2m gene may cause the expression loss.

Microsatellite markers on chromosome 15 were selected to study LOH of the β2m gene. The optimal marker is located within the gene or in close proximity. The locations of the two microsatellite markers flanking the β2m gene suggested by Ramal et al. 2000 [19, 20] (D15S126 & D15S209) should be reconsidered in sight of new information on the human genome. The different microsatellite databases (Marshfield, G3, GB4, Genethron, Genemap 99) do not consistently agree at the position of a microsatellite marker in relation to a certain gene. Therefore, the positions and number of microsatellite markers suitable to study β2m LOH were re-evaluated and expanded [17]. In case of the β2m gene, no informative marker can be found in the gene. Therefore, the question remains whether the gene is lost when LOH is detected. However, as indicated in Table 3, tumors show LOH for the majority of microsatellite markers. Because these markers cover a large chromosomal region, including the β2m gene, it indicates that a large chromosomal region is lost instead of only the β2m gene itself. LOH and CGH studies on HNSCC lesions also demonstrate that genomic alterations cover large regions in these tumors as well as metastases [25-27].

For the majority of the HNSCC lesions, the centromere 1 as well as the centromere 15 FISH results differ between the two tumor fields within a tumor. The percentage of loss or gain is a mean result of the heterogeneous tumor and therefore does not reflect the gene copy number. Microdissection enables the selection of small tumor fields for analysis, although it is clear that one tumor field is not representative for the overall tumor. Even the microdissected field can be heterogeneous. Although normal stromal cells are excluded and the percentage of loss or gain is increased after microdissection, heterogeneity and allelic imbalance can explain why not always a loss percentage of 100% is obtained. In case a tumor has gain instead of loss, both chromosomes will still be present and LOH can never be 100% even after microdissection of a homogeneous tumor field. Moreover, based on LOH data only, a wrong conclusion will be made regarding gene or chromosome copy number: instead of loss, as indicated by the LOH data, a tumor has gain of a genomic region. It is clear that microsatellite marker LOH data are difficult to interpret and is not conclusive for β2m gene copy number in these heterogeneous HNSCC lesions.

In some cases, chromosome 15 FISH analysis can not determine the exact β2m copy number because gene specific loss or gain can not be detected. However, our LOH results show that in tumors with LOH, a large genomic region is lost (Table 3). Other LOH and CGH studies also demonstrate that genomic alterations cover large regions [25-27]. Although it is not likely that specific β2m loss occurs frequently in these HNSCC lesions, it can not be excluded. Therefore, in a tumor with a normal chromosome 15 FISH distribution, specific β2m gene loss may remain undetected by chromosomal FISH analysis. However, only tumor field HN36B and HN26A show a normal chromosome 15 distribution. All other tumors show an affected chromosome 15 copy number. We are currently developing β2m gene specific FISH, which is technically more difficult than chromosomal FISH, to study β2m specific loss in those cases with a normal chromosome 15 distribution. Chromosomal FISH analysis suffices to demonstrate that LOH analysis can not discriminate between loss and gain. Moreover, the heterogeneous aneuploidy in these HNSCC lesions for chromosome 15 and 1 is clearly indicated.

Although chromosome 1 is affected in HNSCC lesions, the median percentage per FISH signal category of all tumors are approximately the same as in centromere 15 FISH. This gives a good indication of the distribution of FISH signals in an average tumor. However, tumors show loss and gain of chromosome 1. Aneuploidy is thus not only limited to chromosome 15 in HNSCC lesions. During tumorigenesis, a cascade of genomic alterations like mutations and deletions occur [28]. It has been demonstrated that chromosomal deletions of 3p, 5q, 9p and amplification of 3q take place early in tumor development. Undifferentiated tumor cells show even more genomic instability throughout the complete genome. Loss of chromosomal region 15q15-q21, where the β2m gene is located, appears to be a late event because it

is first detected in lymph node-positive metastasizing HNSCC lesions [25-27]. In concordance with these publications, nine of the eleven primary tumors in our study with an aberrant chromosome 15 copy number are metastasized.

Moreover, chromosome copy number does not appear to be the same throughout a tumor. To understand what effect aneuploidy and heterogeneity have on tumor progression and therapy, aneuploidy of β2m, HLA genes and HLA related genes need to be studied in detail [29-32]. Our study demonstrates that microsatellite marker studies are not conclusive regarding the copy number of a gene or genomic region.

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Chapter 5

High Level of Aneuploidy of Chromosome 6 by FISH Analysis of Head and Neck Squamous Cell Carcinoma: Limited Applicability of LOH Analysis to Define HLA Loss.

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Abstract: Human Leukocyte Antigen (HLA) Class I molecules are essential for tumor cell recognition by cytotoxic T cells of the adaptive immune system. Loss of HLA expression provides tumor cells with an escape mechanism to evade the immune system and thus immune therapy. Therefore, HLA loss, and in particular loss of heterozygosity (LOH), is frequently studied in tumors using microsatellite marker LOH analysis. Because LOH analysis detects any allelic imbalance and not just allelic loss, we evaluated the LOH analysis in nine head and neck squamous cell carcinoma (HNSCC) using fluorescence *in situ* hybridization (FISH). These tumors were selected out of 53 HNSCC based upon the HLA Class I immunohistochemical staining and LOH data. FISH analysis showed that only two tumors with LOH and one without LOH indeed had loss and a normal chromosome 6 copy number, respectively. Strikingly, for the remaining six tumors, LOH analysis did not reflect the genome HLA copy number. We demonstrated that LOH analysis can not distinguish loss from gain and that the HLA region is not homogeneously affected within a tumor. Tumor heterogeneity and complex aneuploidy in tumors hinder a straightforward interpretation of microsatellite marker analysis. For immune therapy strategies in cancer patients, knowledge of the HLA expression on tumor cells is essential, to which LOH analysis has a limited contribution.

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5.1 Introduction

The human leukocyte antigens (HLAs) are a key component in human tumor immunology. Presentation of tumor associated antigens (TAA) by HLA class I/ β2-microglobulin (β2m) complexes to the T-cell receptor (TCR) of cytotoxic T lymphocytes (CTLs) allows specific targeting of neoplastic cells by the adaptive immune system [1]. Loss of HLA expression not only affects the efficacy of the adaptive immune response in tumor eradication, but also hampers the applicability of immune therapy [2-5].

To understand the effect of HLA expression-loss in tumors better, HLA loss is categorized in loss-phenotypes [2]. Using locus specific anti-sera, total-loss and HLA locus-loss can be categorized. However, the most frequently occurring phenotypes, haplotype-loss and allelic-loss phenotypes, are more difficult to characterize because of the current lack of sufficient HLA allele-specific anti-sera. Therefore, in frame of the 13th IHWG HLA and Cancer component, workshop-defined antibodies are tested to evaluate HLA-loss phenotyping in different tumors. Additionally, when allele-specific antibodies are available, tumors need to be typed for HLA to test them with the appropriate antibodies. It should be noted that there are still limitations to the use of anti-sera because of antibody cross-reactions between the highly resembling HLA alleles [6].

It is known that genomic loss, and in particular loss of heterozygosity (LOH), in the HLA region and the β2m gene is a common cause of HLA expression-loss in tumors [7-11]. Therefore, LOH analysis is a frequently applied method by which HLA locus-loss and, in particular, allelic-loss can be determined. LOH of HLA genes results in a diminished variety of HLA molecules that can be expressed. This affects the diversity of TAA that can be presented to the TCR of CTLs. Loss of both alleles of a gene (e.g. β2m) has a total deletion effect, whereas LOH results in the genomic presence of only one intact allele. According to the "second-hit" theory, a mutation in the remaining allele may eliminate gene expression. Therefore, tumors that show LOH for HLA genes not only lack one of the two alleles, but also are more susceptible for the effect of a second mutation.

Microsatellite markers are often used to study LOH patterns in tumors [7, 12, 13]. LOH is ascribed to the tumor when microsatellite markers show a decrease of at least 25% of one allele relative to the second in the tumor, compared to the ratio in peripheral blood lymphocytes (PBLs) [14]. However, it should be noted that microsatellite marker analysis detects not merely LOH, but also any other allelic imbalance [15]. The complication is that one can not distinguish between the gain of one allele and the loss of the other allele. Besides, a normal microsatellite marker ratio does not necessarily mean that there are only two alleles present. It indicates that both alleles are present in the same ratio (e.g. two copies, three copies, etc.). Therefore, microsatellite analysis is insufficient, in many cases, to characterize

genomic loss. It should be noted that LOH analysis can not accurately determine the HLA expression loss on the cell surface. One can not discriminate between locus loss, allelic loss, haplotype loss or a combination of these, especially in cases with complex loss patterns [14]. Moreover, it remains questionable whether loss of a microsatellite marker is representative for the expression of adjacent genes.

In this study, we conducted chromosome 6 fluorescent *in situ* hybridization (FISH) on head and neck squamous cell carcinoma (HNSCC) to determine whether HLA microsatellite marker LOH analysis always represents true LOH or is affected by allelic (im-)balance and tumor heterogeneity. Because FISH is a very labor-intensive technique, we selected nine tumors from a panel of 53 HNSCC for which we performed LOH analysis with microsatellite markers TAP1CA, C125 and D6S265. It has been demonstrated that these markers, among others, are representative for HLA LOH analysis [16, 17]. As we have shown before, a small subset of markers is representative for a large genomic region [15]. The HNSCC were screened for HLA Class I expression by monoclonal antibody (mAb) W6/32 immunohistochemistry. HLA-A (HC-A2) and HLA-B/C (HC-10) immunohistochemistry was performed in a previous study [18]. Tumors with a heterogeneous HLA expression were excluded for FISH analysis because of the associated complexity of LOH data interpretation. Three "normal" tumors without LOH were included as well as six "aberrant" HNSCC with LOH to study the genomic loss by FISH.

5.2 Materials and Methods

5.2.1 Tissue Samples

The 53 HNSCC specimens used in this study were obtained surgically at our department between 1996 and 2001 and underwent standard tissue processing. For each tumor sample, frozen as well as paraffin-embedded tumor tissue is available. Therefore, these preparations are directly comparable and specific for the tumor. For LOH analysis, we used high-quality DNA isolated from frozen tumor tissue instead of the more fragmented DNA that would be obtained from paraffin-embedded tissue. HLA immunohistochemical staining and microsatellite LOH studies were performed on all 53 tumors. Based on the immunohistochemical staining and the LOH data, nine (five oral-cavity carcinomas, two larynx tumors and two hypopharynx carcinomas) of the 53 HNSCC were selected for FISH to evaluate the HLA LOH analysis. These included six tumors with LOH and three without LOH.

5.2.2 Immunohistochemical Staining

Immunohistochemical staining was performed on 4 µm-thick frozen sections of 53 HNSCC. Sections were mounted on glass slides. The mAb W6/32 was diluted 1:100 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA)

and 0.2% sodium azide. For immunohistochemical staining, the three-step biotin-streptavidin-peroxidase technique was used with 3,3'-diaminobenzidine.4HCl (DAB) as chromogen. Sections were analyzed by two independent observers. Discrepancies were re-evaluated in the presence of a third independent observer.

5.2.3 Loss of Heterozygosity

LOH analysis was carried out on 53 HNSCC as described previously [15]. In short, DNA of high quality was isolated from PBL and HNSCC cryostat tissue sections. To detect LOH of the HLA region, we used microsatellite markers TAP1CA, C125 and D6S265 for the centromeric, central and telomeric part of the HLA region, respectively. Genescan signals over 6,000 mean fluorescence intensity (MFI) or below 100 MFI are considered to be not reliable. LOH was calculated as described by Ramal, *et al.* 2000 [16].

5.2.4 Fluorescence in situ Hybridization

Six µm-thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks and mounted on silan-coated glass slides. The sections were baked overnight by incubating at 58°C, deparaffinized in xylene, rehydrated in an ethanol series and air-dried. Of each tumor, one section was stained haematoxylin-eosin (HE) for morphologic review. For FISH, sections were pepsinized for 10 min at 37°C in 500 U/ml pepsin in 0.1 M glycin/HCl pH 2.0, washed and autoclaved in PBS. After the slides reached room temperature, they were dehydrated in an ethanol series and air-dried.

For chromosome 6 FISH, a centromere 6 probe (ATCC, Rockville, MD, USA) was digoxigenin-labeled using a nick translation kit (Roche, Nutley, NJ, USA) according to the manufacturer's manual. The nicked digoxigenin-labeled probes were checked for size (ideally 100 bp - 600 bp) on a 1.5% agarose gel and precipitated using 1 volume sonicated haring sperm DNA (HSD), 0.1 volume 3 M sodium acetate pH 5.2 and isopropanol. The probe was dissolved in 1 mM Tris 0.1 mM EDTA to a final concentration of 20 ng/µl. The probe hybridization mixture (200 ng probe in 40 µl hybridization buffer containing 50% formamide, 2× SSC, 10% dextran sulfate) was boiled for 5 min and subsequently cooled on ice for 5 min. After pipetting the hybridization mix onto each tissue section and applying coverslips, co-denaturation of target and probe DNA was achieved by placing the slides on a hot plate for 10 min at 98°C, followed by 5 min on ice. The slides were incubated overnight in a moist environment at 37°C, washed (50% formamide, 2× SSC, 0.05% Tween 20) for 5 min at RT, 10 min at 45°C and subsequently in 2× SSC, 0.05% Tween 20 for 15 min at RT. Stringency wash was followed by washing in 4× SSC/ 0.05% Tween 20 and blocking with 100 µl block buffer (5% nonfat milk in 4× SSC/ 0.05% Tween 20) for 10 min. Probe hybridization was detected by incubation with 100 µl

mouse-α-digoxigenin (diluted 1:50 in block buffer) for 1 hour followed by washing (0.05% Tween 20/PBS). Subsequently, the tissue sections were blocked with 10% heat-inactivated normal human serum (NHS) in PBS for 10 min and incubated with 1:40 rabbit-α-mouse-FITC in 10% NHS/PBS for 30 min. After washing with 0.05% Tween 20/PBS, the samples were blocked again using 10% NHS/PBS for 10 min and incubated with 1:100 goat-α-rabbit-FITC in 10% NHS/PBS for 30 min. Tissue sections were washed in PBS and nuclei were stained using 100 µl propidium iodide (1:10,000 in PBS) for 20 s. Finally, the sections were washed twice in 0.05% Tween 20 in PBS and covered with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and a coverslip. Of each HNSCC slide, two tumor fields within a tumor

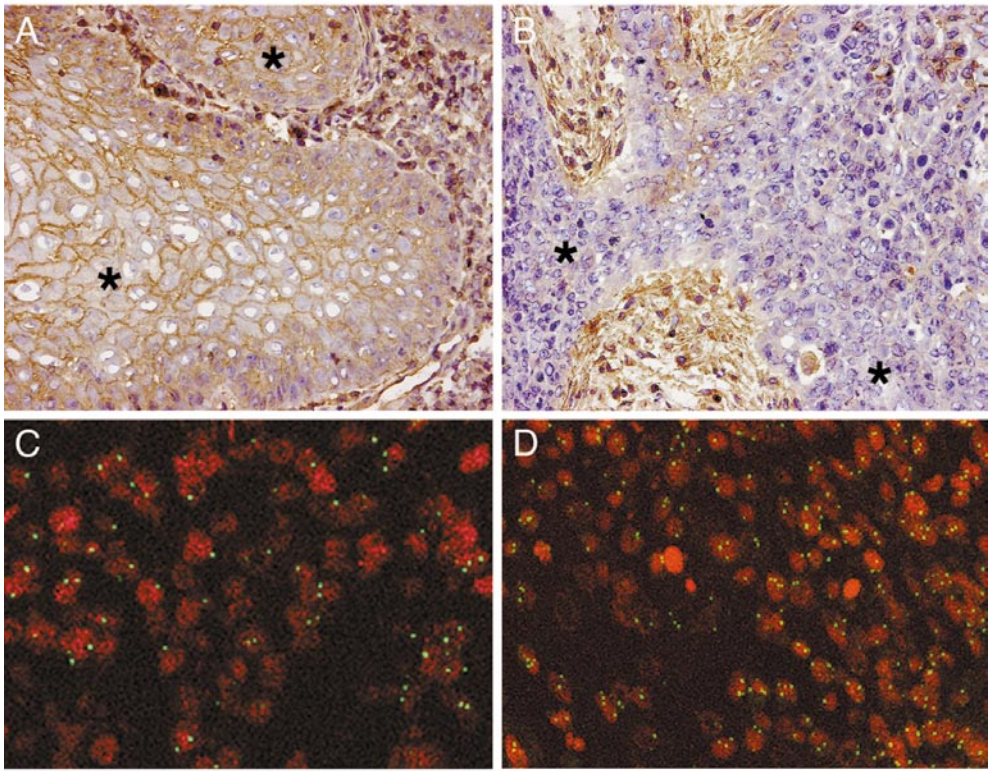

Figure 1. Peroxidase HLA class I (W6/32) immunostained frozen sections of head and neck squamous cell carcinoma (100×) with (A) positive staining and (B) negative staining of the tumor cells (*). Centromere 6 fluorescence *in situ* hybridization examples of head and neck squamous cell carcinoma showing (C) loss and (D) gain of chromosome 6. Nuclei were stained red using propidium iodide while the centromere 6 probe was visualized in green using FITC-labeled α-digoxigenin (1:10,000). Overlapping nuclei were not included in the analysis.

(field A and field B) were analyzed separately by two independent persons using a Leica DMR fluorescence microscope. The number of fluorescent signals per cell was categorized into five groups: no signal, one signal, two signals, three signals and more than three fluorescent signals. On average, 250 cells were counted with a minimum of 150 cells. Overlapping nuclei were not included in the analysis. Hybridization efficiency was tested on PBL spots and two control tissues (tonsil) were included.

5.3 Results

5.3.1 Immunohistochemical Staining & Microsatellite Analysis

Monoclonal antibody W6/32 was used to conduct immunohistochemical staining for HLA Class I on 53 HNSCC. The staining patterns of the tumor samples were categorized as positive, negative or heterogeneous staining. Examples of a positive and a negative staining are shown in Figures 1A and 1B, respectively. Only tumor HN29 had no HLA Class I expression; the other HNSCC showed some degree of HLA Class I expression. Because antibody W6/32 detects all HLA class I molecules, locus- and allelic-loss can not be excluded. The HLA immunohistochemical staining results of the nine tumors selected for FISH, together with the results of the HLA microsatellite and FISH analysis, are summarized in Table 1. LOH was ascribed to the tumor when microsatellite markers showed a decrease of at least 25% of one allele relative to the second in the tumor, compared with the ratio in peripheral blood lymphocytes (PBLs) [16]. The raw LOH data are shown in Table 2. In this series of nine tumors, the loss percentage of tumors with LOH was at least 50% for all microsatellite markers, except for HN48 and HN23, which showed a microsatellite loss percentage of approximately 35%. Tumor HN23 was homozygous for TAP1CA and did not show loss for marker D6S265. This tumor may therefore have only partial loss of the HLA region. In general, LOH in the tumors was detected with all three microsatellite markers, indicating that genomic loss is widely dispersed throughout the HLA region. Therefore, these three microsatellite markers suffice to study the loss of the HLA region.

5.3.2 Fluorescence in situ Hybridization

Stacked CSLM images of a tumor with loss and another tumor that showed amplification of chromosome 6 are depicted in Figures 1C (HN04) and 1D (HN49), respectively. Figure 1C shows nuclei (red) with predominantly zero and one FISH signal (green). In Figure 1D, in addition to nuclei with two FISH signals, many nuclei have three and more signals. The FISH distribution of two tumor fields, A and B, within one HNSCC was combined when the distribution was not significantly different (Chi-square $p<0.05$), indicated by an asterisk (*) in Table 1[c]. An overview of the chromosome 6 FISH results is given in Figure 2.

Table 1. Data overview of the nine HNSCC selected for FISH analysis

			FISH Chromosome 6	
Tumor	W6/32 IH [a]	LOH STR [b]	Field [c]	Loss/Gain [d]
HN21	+	L	A B	N L
HN23	+	L	A B	G L
HN48	+	L	A B	L G
HN29	-	L	A* B*	L
HN04	+	L	A B	L L
HN49	+	L	A B	G G
HN35	+	NL	A B	G G
HN10	+	NL	A* B*	L
HN42	+	NL	A* B*	N

a) immunohistochemical HLA class I staining (W6/32), positive (+) and negative (–) staining

b) microsatellite marker LOH analysis, LOH (L), no LOH (NL)

c) for each HNSCC, two tumor fields (A and B) were analyzed for FISH. Asterisk indicates no statistical difference of chromosome 6 FISH distribution between tumor fields

d) chromosome 6 FISH in tumor cells, normal (N), loss (L) and gain (G)

Hybridization efficiency of the chromosome 6 FISH was tested on PBL spots (data not shown). As a result of paraffin-embedded tissue sectioning for FISH, a certain percentage of the nuclei in the sections were truncated. This phenomenon influences the distribution of FISH signals. As a control for FISH in tissue sections, two tonsils were used. Additionally, an area of lymphocytes in tumor HN49 was used as internal control (indicated by LymphoC in Figure 2). The sum of chromosome 6 FISH categories “zero signals per nucleus” and “one signal per nucleus” is below 35% in the controls (Figure 2). The categories “three signals per nucleus” and “more than three signals per nucleus” vary in the controls. Overall, less than 20% of the cells, representing (truncated) proliferating cells, show three and more FISH signals per nucleus. Based on these data, the criteria were set as follows: chromosome 6 loss was assigned when more than 35% of the cells have zero or one FISH signal while gain of chromosome 6 was assigned when at least 20% of the cells showed three and more FISH signals. These criteria are in concordance with those of our previous study [19].

Table 2. Overview of the raw LOH data

		LOH % [b]		
Tumor	Tumor % [a]	TAP1CA	C125	D6S265
HN21	80	56,8	58,2	50,9
HN23	70	H	32,9	5,7
HN48	60	27,2	30,3	38,9
HN29	60	67,6	80,3	68,1
HN04	60	H	50,2	49,6
HN49	70	54	54,9	H
HN35	70	9,1	8,5	H
HN10	80	10,8	22,4	1,1
HN42	60	11	3	H

a) tumor percentage of the cryostat tissue sections used for LOH analysis

b) LOH percentage of the individual microsatellite markers used to study LOH of the HLA region. Homozygous microsatellite markers are indicated by (H) for each sample

The chromosome 6 FISH distribution of tumor HN42 is comparable to the controls. Microsatellite analysis did not detect LOH and immunohistochemical staining with W6/32 was positive. HLA did not appear to be affected in this HNSCC and LOH analysis was in concordance with the results of the other techniques.

Three tumors (HN29, HN04 and HN10) clearly showed loss of chromosome 6. LOH analysis detected this loss in HN29 and HN04. In case HN10, the LOH results did not correspond to the FISH results of the two homogeneous tumor fields. Because the loss of one chromosome 6 was clearly indicated by FISH, the lack of LOH and the positive immunohistochemical staining could possibly have arisen from intrachromosomal duplication or interchromosomal recombination of the HLA region. This cannot be detected by centromeric FISH. However, the positive HLA expression may also result from the remaining HLA alleles, indicating that microsatellite analysis is not valid in this tumor. The only way to determine whether both HLA haplotypes are still present and expressed is by using allele-specific anti-sera. Unfortunately, the HLA alleles of this sample cannot be discriminated by the currently available mAbs.

In tumors HN23 and HN48, FISH showed a heterogeneous chromosome 6 distribution. Loss as well as gain of chromosome 6 was detected in the distinct tumor fields. For both HNSCC, immunohistochemistry showed a positive staining and microsatellite LOH. In these cases, both loss and gain contribute to the allelic imbalance detected by microsatellite analysis, whereas LOH analysis suggests that there is only loss. Microsatellite LOH can not distinguish between loss and gain and is therefore not valid in these HNSCC.

FISH detected gain of chromosome 6 in HN49 and HN35, while microsatellite analysis showed “LOH” and “no LOH”, respectively. In both cases, chromosome 6, and therefore HLA, is amplified instead of lost or normally present. Again, microsatellite analysis is not representative for the genomic HLA copy number.

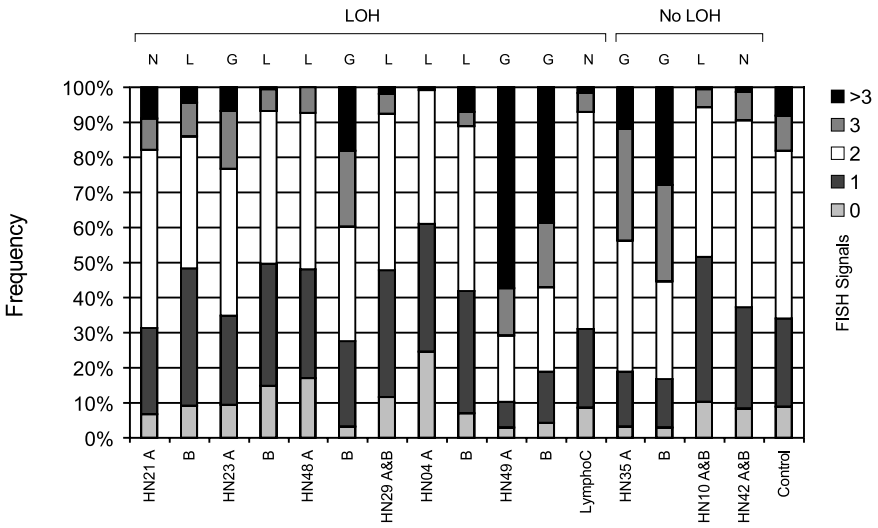

Figure 2. Cumulative presentation of centromere 6 FISH frequencies of tumor field A and B of nine HNSCC. Control, LymphoC and HN42 are examples of a "normal" FISH distribution while HN10 and HN04 clearly show loss of chromosome 6. HN35 and HN49 are examples of HNSCC with gain of chromosome 6. Gain (G), loss (L) or normal (N) chromosome 6 distribution is indicated above each bar.

Finally, HN21 showed a normal FISH distribution for tumor field A, whereas the other tumor field has loss of chromosome 6. Although LOH was detected, it does not completely reflect the HLA copy number in this HNSCC.

Based on these results (as summarized in Table 1) it can be concluded that: i) chromosome 6 ploidy is affected in eight of nine HNSCC; ii) gain as well as loss (complex aneuploidy) has been identified in these tumors; iii) microsatellite analysis can not distinguish loss from gain; iv) chromosome 6 ploidy can be heterogeneous within a tumor; v) in six out of the nine HNSCC, LOH analysis is not representative for the HLA copy number.

5.4 Discussion

We have studied genomic loss of the HLA region in a selection of nine out of 53 HNSCC using immunohistochemistry, microsatellite marker analysis and chromosome 6 FISH. Immunohistochemistry is an excellent technique to study protein expression in tumors. However, the current lack of sufficient HLA-allele-specific anti-sera and the likelihood of cross-reactions between resembling HLA alleles limits the applicability. The W6/32 staining is merely a rough indication of HLA class I expression in a tumor. Normal, heterogeneous or negative expression can be detected, but no distinction can be made between the expression of the classical

HLA class I genes. We applied mAb W6/32 and used our previous HC-A2 (HLA-A) and HC-10 (HLA-B/C) immunohistochemistry data to screen the HNSCC for heterogeneous HLA expression. It should be noted that antibodies HC-A2 (HLA-A) and HC-10 (HLA-B/C) cross-react, so locus-loss can only be determined when the HLA class I typing is known and cross-reactivity can be excluded [6] or allele specific antibodies can be used [20]. The interpretation of LOH data in heterogeneous tumors is complicated since the loss percentage is a result of the different tumor fields within the tumor section. Ideally, combined analysis by immunohistochemical staining and LOH is most informative. Microdissection on a immunohistochemical stained tissue section provides a solution, but this is technically not routinely applicable. In a heterogeneous tumor, microdissection can be applied to study all tumor fields separately, but two tumor fields suffice to define tumor heterogeneity. Since heterogeneous tumors display complex patterns of combined factors that influence the HLA expression, we excluded tumors with heterogeneous HLA class I expression in this study.

Only in tumor HN29, a combination of mutations, or gene methylation, and LOH have resulted in a total loss of HLA class I expression. All other tumors showed some degree of HLA class I expression. However, locus-loss and allelic-loss can not be excluded. Because of the affected genomic HLA region, the antigen presentation by HLA class I is suboptimal, which hampers the CTL immune response in these HNSCC. Because HLA expression is not completely lost, a NK cell response is not likely to occur. Therefore, the balance between HLA expression and loss may provide tumors an "escape-window" to evade both NK cell and CTL immune responses.

For microsatellite marker analysis, we used the LOH detection cut-off point of 25% as described by Ramal *et al.* [14, 16]. The loss percentages of the markers TAP1CA, C125 and D6S265 where on average 50%. Therefore, the utilization of microdissection was not of additional value for LOH analysis. However, it should be noted that when microdissection is applied, a 100% loss is rarely found due to heterogeneity of the microdissected tissue or allelic gain rather than loss. In both cases, the two alleles are still present in a disproportional ratio and can be detected by microsatellite analysis. The heterogeneity of HNSCC and the complex aneuploidy in the tumor cells hamper the interpretation of the LOH analysis [15]. Intra-tumor heterogeneity is not limited to HNSCC but has also been described in other tumors [21]. This emphasizes the urge to validate the LOH microsatellite analysis in heterogeneous tumors. In homogeneous tissues, LOH analysis is sufficient to study allelic (im)balance. However, if one wants to differentiate between loss and gain, additional techniques like FISH are necessary.

The HLA region is a 4 Mb cluster on chromosome 6p, thus loss or gain of the chromosome is representative for the HLA cluster. FISH with a centromeric probe can only detect LOH by chromosome loss. Deletion, gene conversion and mitotic

recombination will remain undetected and might be elucidated by gene specific FISH or Fiber-FISH. Chromosome 6 FISH may therefore underestimate the HLA copy number. The microsatellite markers we used cover the complete HLA region and are uniformly affected, indicating that the total HLA region, and even beyond, is likely to be affected. Besides, other LOH and CGH studies demonstrate that large genomic regions are affected rather than specific HLA loci [15, 22]. Taking all this into account, chromosome 6 FISH suffices to demonstrate that microsatellite marker analysis can not distinguish between the variety of allelic imbalances.

The chromosome copy number is affected in a certain percentage of cells because of proliferating tumor cells and truncated nuclei in the tissue sections for FISH analysis. We used the chromosome 6 distribution in control tissues and areas of lymphocytes in tumors (e.g. HN49) to set the criteria to define chromosomal loss (35%) and gain (20%). This is the chromosome 6 FISH distribution in normal tissue with a majority of diploid cells. Although FISH analysis indicated gain and a normal chromosome 6 distribution in HN23A and HN21A, respectively, the difference between the two tumor fields is arguable. In HN23A, 23% of the tumor cells show three and more FISH signals per nucleus, which is only 3% more than the criteria for gain. Therefore, this tumor field is a borderline case for which the chromosome 6 distribution should be described as "normal with minor gain". However, in all other cases, loss or gain is clearly indicated by the FISH analysis.

In six of the nine HNSCC, the HLA microsatellite LOH results did not correspond with the chromosome 6 copy number as detected by FISH. In cases HN49 and HN35, where chromosome 6 gain is mistaken for LOH and not detected by microsatellite analysis respectively, the flaw in LOH analysis is most clear. Although microsatellite analysis is an easy technique, it can not correctly detect allele copy numbers. Gain can not be distinguished from loss in either allelic imbalance or allelic balance. Only a loss percentage of 100% is informative and represents true LOH. All other loss percentages can represent allelic (im)balance or tumor heterogeneity. Although LOH detects alleles, it can not distinguish individual cells, whereas FISH looks at individual nuclei, but can not distinguish alleles. To discriminate between loss and gain, or to define tumor heterogeneity, additional techniques like FISH are required.

In cancer patients, it is of critical importance to determine the HLA expression in tumor cells to be able to develop and apply effective immune therapy strategies. Considering our data, microsatellite marker LOH analysis provides a very limited contribution. Although FISH analysis gives a good insight into the genomic status of the HLA region, it is not really suitable for routine use in a clinical setting. Moreover, genomic presence of an HLA gene is no guarantee for cell surface expression. Therefore, immunohistochemistry remains the best option to characterize HLA expression on tumor cells. However, a lot of effort and research will be required to develop sufficient allele-specific anti-sera.

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Chapter 6

HLA Class I Allele-specific Expression-loss in Head and Neck Squamous Cell Carcinoma and Corresponding Lymph Node Metastases.

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Abstract: Human leukocyte antigen (HLA) expression is crucial for the elimination of tumor cells by the immune system and immunotherapy. Activated T cells directed against tumor associated antigens (TAA) are fully capable of recognizing and eradicating neoplastic cells. Therefore, HLA expression-loss is considered to be a main factor in tumor development. We report for the first time HLA-A and HLA-B allele-specific expression analysis by immunohistochemical staining of fresh tumor tissue and 9 lymph node metastases of 15 HNSCC patients. Heterogeneous HLA expression and HLA expression-loss was detected in 13 tumor patients. Approximately 50% of the tumors had allele-specific expression-loss which would have remained undetected using HLA monomorphic and locus-specific antibodies. HLA-B expression was more frequently affected than HLA-A expression. Strikingly, all five HNSCC patients with the HLA-B8/DR3 haplotype showed aberrant expression of HLA-B8. This suggests preferential negative selection of HLA-B8 expressing tumor cells in tumor patients with the autoimmune disease associated HLA-B8/DR3 haplotype. In the majority of the HNSCC patients, HLA allele-specific expression-loss differed between primary lesions and metastases. This is important for the efficacy of immunotherapy in these patients. It can be concluded that it is crucial to study HLA expression at the allele-specific level of primary lesions and metastases. It increases and refines our knowledge of HLA expression-loss in tumorigenesis which will improve the development of specific immunotherapy.

Submitted

6.1 Introduction

In the past decades, T cell-based immunotherapy has become increasingly important in improving cancer treatment [1-3]. Activated T cells directed against tumor associated antigens (TAA) are fully capable of recognizing and eradicating neoplastic cells [4]. In general, TAA are derived from mutated or overexpressed intracellular proteins and are presented to CD8+ T cells on the tumor cell surface in association with HLA class I molecules [5, 6]. The efficacy of immunotherapy is therefore largely dependent on the expression of the appropriate HLA class I antigen on the tumor cell [7]. HLA loss or downregulation is a frequent event in tumor development and represents a mechanism by which tumor cells evade immune surveillance and become resistant to immunotherapy [8, 9]. Not surprisingly, many studies have been conducted to elucidate the role of HLA expression-loss in various tumors and tumor cell lines [10-14]. HLA expression-loss is defined by the following loss-phenotypes: phenotype 1, total HLA loss; phenotype 2, HLA haplotype loss; phenotype 3, HLA locus loss; phenotype 4, HLA allelic loss and phenotype 5, compound loss [8]. Total HLA loss, haplotype loss and locus loss can be determined with monomorphic and locus-specific antibodies, but allelic loss and compound loss can not be adequately identified. The lack of sufficient HLA allele-specific monoclonal antibodies (mAbs) has been the bottle-neck in characterizing HLA expression-loss. Since TAA are presented by restricted HLA class I alleles, it is crucial to study HLA downregulation or expression-loss at the allele-specific level. HLA loss is most frequently caused by mutations in, and genomic loss of the HLA region [15]. To circumvent the lack of sufficient HLA allelic product-specific mAbs, alternative techniques, such as loss of heterozygosity (LOH) analysis, comparative genomic hybridization (CGH) and fluorescence *in situ* hybridization (FISH), were developed to predict HLA loss [16-19]. However, the major drawback of these and other DNA-based techniques is that they are not fully representative for HLA cell surface expression [18, 19]. To adequately study the role of HLA class I expression-loss in immune evasion by tumor cells, HLA allelic product-specific antibodies are required.

Recently, allele-specific HLA class I loss was studied in a limited number of head and neck squamous cell carcinoma (HNSCC) cell lines as well as lung cancer cell lines by flow cytometric analysis [14, 20]. Although these studies shed the first ray of light on the role of HLA expression-loss in tumor development, the use of cell lines entails the likelihood that HLA antigen alterations might have developed during prolonged *in vitro* culture. Moreover, due to preferential expansion of a specific sub-population, cell line cultures often represent a homogeneous sub-population of the heterogeneous *in situ* tumor. These uncertainties are eliminated when fresh tumor tissue is studied. Heterogeneity of HLA expression within the tumor can be detected in fresh tumor tissue sections using immunohistochemical staining [21]. This is of crucial importance, since it is suggested that selective pressure by the immune system

or by immunotherapy stimulates the generation of malignant lesions with HLA class I defects [9]. This allows the tumor to develop escape variants. Heterogeneous HLA expression within a tumor may reflect the presence of escape variants *in vivo*.

The availability of a panel of polymorphic HLA specific mAbs, that were adapted for immunohistochemical staining, enabled the study of HLA-A and HLA-B allele-specific expression in fresh tissue sections of 15 HNSCC patients. Paucity of HLA-C allelic product-specific mAbs precludes the study of HLA-C expression-loss. Lymph node metastases of 9 out of the 15 HNSCC patients were included to study the HLA expression-loss difference between the primary tumor and its metastasis. To our knowledge, this is the first time HLA allele-specific expression is characterized in fresh tumor tissue and corresponding lymph node metastases at the level of antigen expression. Expanding this line of investigation will increase our insight in HLA expression-loss in tumors and will provide knowledge to improve selective immunotherapy.

6.2 Materials and Methods

6.2.1 Tissue Samples

Tumor tissue and lymph node metastases were obtained from 15 HNSCC patients that were surgically treated for this tumor at the departments of craniomaxillofacial surgery and otorhinolaryngology at the University Medical Centre Utrecht between 1996 and 2001. Although all patients were diagnosed with metastases, snap-frozen lymph node metastases were only available from 9 patients. The 15 HNSCC patients were selected out of a panel of over 100 HNSCC patients based on their HLA-A and HLA-B typing for which appropriate mAbs were available.

6.2.2 HLA Typing

Over 100 HNSCC patients were previously typed at intermediate/low resolution for HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ using the sequence specific priming (SSP) kit from Biotest (Dreieich, Germany). SSP typing was performed according to the manufacturer's instructions using DNA isolated from peripheral blood of the tumor patients by the salting-out method [22]. Sequencing-based high-resolution typing (SBT) was performed on the same DNA for HLA-A, HLA-B and HLA-C: HLA-A and HLA-B (Abbott BV, Germany) and HLA-C [23]. High resolution HLA typing of sample HN02 was not possible due to low quality DNA. HLA typing data was used for haplotype analysis which allowed us to indicate the most likely haplotypes in the majority of the HNSCC patients [24-26].

6.2.3 HLA Monoclonal Antibodies

Human hybridomas were established from B-lymphocytes of HLA antibody seropositive, multiparous women by EBV transformation, followed by electrofusion and HAT-ouabain selection of antibody secreting EBV lines, and rigorous subcloning. HLA-specificities of mAbs were determined by complement dependent cytotoxicity (CDC) against large (n>240) panels of serologically HLA typed peripheral blood mononuclear cells. Some mAbs react with epitopes shared between several allelic products. HLA mAbs of IgG isotype were purified from hybridoma supernatants by protein A chromatography (Pharmacia, Uppsala, Sweden). Ammonium sulphate cuts (30-50% saturation) of supernatants containing IgM isotype HLA mAbs were dialyzed against PBS and loaded on a High Load 16/26 Sephadex G-75 column (preparation grade, Pharmacia). Fractions with MW exceeding 75 kD were collected, pooled and dialyzed against PBS. Protein concentrations of purified mAb preparations were determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). The purity of IgM-mAb preparations was assessed by PAGE under reducing conditions. Purified HLA-mAbs were labeled with biotine using a biotinylation kit (#21430, Pierce) according to the manufacturer's instructions. The reactivities of biotine-labeled HLA mAbs were validated by flowcytometry. In this validation study, selected (for HLA phenotype) peripheral blood mononuclear cells (5x105) were incubated with 1 μg of biotine labeled HLA-mAbs followed by phycoerythrin conjugated streptavidin and FITC-labeled anti CD3 mAb (both from Becton & Dickinson, Mountain View, CA) and analysed on a FACSCalibur (BD Biosciences - Immunocytometry Systems, San Jose, CA) equipped with CellQuest. All biotine mAbs showed homogeneous, HLA allelic product-specific, staining on CD3 positive cells. Geometric means of PE fluorescence intensity reached at least the third log in fluorescence histograms.

6.2.4 Immunohistochemical Staining

Immunohistochemical staining was performed on 6 μm frozen sections. Sections were mounted on glass slides and dried overnight at room temperature (RT). After fixation in acetone for 10 minutes, slides were incubated for one hour with the appropriate primary biotinylated human HLA mAb at the dilutions given in Table 1. Slides were fixed in 4% phosphate-buffered formaldehyde (Klinipath, Duiven) for 10 minutes and incubated for 15 minutes with peroxidase-blocking solution containing 0.04 M citric acid, 0.12 M sodium phosphate dibasic dehydrate (both from Riedel de Haën, Seelze, Germany), 0.03 M sodium azide and 0.05% perhydrol (both from Merck, Darmstadt, Germany) in deionized water. Subsequently, the sections were incubated for one hour with a mouse-anti-biotin mAb at the dilution 1:400 (DAKOCytomation, Glostrup, Denmark) and subsequently for 30 minutes with PowerVision poly HRP-Anti-Mouse (Immunologic, Duiven, The Netherlands). Staining was visualized by

incubating the slides with 3,3-diaminobenzidinetetrahydrochloride (Sigma-Aldrich, Deisenhofen, Germany) for 10 minutes as a chromogen. Slides were counterstained with Mayer's haematoxylin, dehydrated and coverslipped. Between incubations, sections were washed three times with PBS containing 0.05% Tween 20. All mAbs were diluted in PBS containing 1% BSA and 0.2% sodiumazide. The specificity, isotype and dilutions of the human mAb used for immunohistochemical staining are listed in Table 1. Selection of appropriate allele-specific mAbs was based on the HLA typing results of the patient. As a negative control for background staining, the primary mAb was omitted. Staining was scored as positive, heterogeneous and negative by two independent persons.

Table 1. HLA mAbs for immunohistochemical staining

mAb	Specificity [a]	Isotype [b]	Dilution [c]
SN230G6	A2/B17	IgG1, λ	1:400
SN607D8	A2/A28	IgG1, κ	1:200
OK2F3	A3	IgM, κ	1:100
GV5D1	A1/A9	IgG1, λ	1:200
BVK5C4	A9/A80	IgM, κ	1:200
OK6H10	B15/B21/B56/B35/B72	IgM, κ	1:30
HDG8D9	B51/B35	IgG1, λ	1:200
IND2D12	B15/B35/B21/B70	IgM, λ	1:200
VTM1F11	B27/B7/B60	IgG1, κ	1:400
JOK3H5	B40/B21/B13/B12/B41/B70	IgM, λ	1:200
BVK1F9	B8	IgG1, κ	1:15

a) HLA-A and HLA-B allele-specificity

b) isotype of the mAb

c) dilution for application in immunohistochemical staining

6.3 Results

The selection of 15 patients for this study was based on compatibility of their HLA class I SSP typing results with the panel of available biotine-labeled HLA allelic product reactive human mAbs. This panel enabled study of expression of all HLA-A and HLA-B alleles in the tumors of 8 patients, 3 alleles in 6 patients and 2 alleles in one patient. HLA-A*01 expression in tumor HN15 could not be studied due to cross-reactivity of mAb GV5D1 with the serologically defined HLA-A9 group to which the HLA-A24 antigen belongs.

An overview of tumor characteristics and HLA typing of the HNSCC patients are presented in Table 2. HLA class I high resolution typing was performed to gain

Table 2. Characteristics of the 15 patients with HNSCC

				HLA allele assignment[d]									
Tumor	Age[a]	Sex[b]	Site[c]	A1	A2	B1	B2	C1	C2	DR1	DR2	DQ1	DQ2
HN01	59	F	Oral cavity	030101	**3201**	5108	**15010101**	1602	**030301**	11	13	03	06
HN02	44	M	Hypopharynx	**02**	32	**40**	***51***	**03**	***15***	**13**	***01***	**06**	***05***
HN03	47	M	Oropharynx	**010101**	010101	**0801**	3801	**070101**	120301	**03**	13	**02**	06
HN04	65	M	Oropharynx	**010101**	***02010101***	**0801**	***070201***	**070101**	***070201***	**03**	***15***	**02**	***06***
HN05	59	M	Oropharynx	02010101	**02010101**	510101	**4002**	020202	140201	14	**13**	05	**06**
HN06	54	M	Oral cavity	***010101***	**02010101**	***0801***	**070201**	***070101***	**070201**	***03***	**15**	02	**06**
HN07	56	M	Oral cavity	02010101	**03010101**	510101	**070201**	070201	**070201**	04	13	06	03
HN08	81	F	Oral cavity	**010101**	02010101	**0801**	***3701***	**070101**	***0602***	**030101**	030101	**02**	03
HN09	43	M	Hypopharynx	**02010101**	24020101	**070201**	***15010101***	**070201**	***030403***	12	***15***	**06**	03
HN10	41	M	Oral cavity	**02010101**	***02010101***	**400102**	***510101***	**030401**	***070201***	**13**	14	**06**	05
HN11	51	F	Oral cavity	**02010101**	3201	**4002**	***0801***	020202	***070101***	**13**	***03***	n.d.	n.d.
HN12	51	F	Oropharynx	010101	3301	1402	510101	0802	140201	010101	010201	n.d.	n.d.
HN13	70	M	Oral cavity	**02010101**	***02010101***	**15010101**	***4001***	**030301**	***030301***	130201	***130101***	06	***06***
HN14	69	M	Hypopharynx	010101	***02010101***	510101	***15010101***	140201	***030401***	04	08	03	04
HN15	43	M	Oral cavity	**010101**	***24020101***	**570101**	***510101***	**0602**	***150201***	13	***07***	06	***03***

a) age in years at the time of tumor resection

b) sex, (M) male and (F) female

c) site of the primary tumor

d) HLA typing results with the two theoretically defined haplotypes (A1, B1, etc. & A2, B2, etc.) in bold and bold italics, respectively. Extended SBT for exon 1 excluded the B*15N allele

information on the allelic specificity of the mAbs. Using the HLA class I and class II typing, we were able to define the most likely HLA haplotypes which are indicated in bold and italics. Five (HN03, HN04, HN06, HN08, HN11) of the 15 HNSCC patients have the HLA-B8/DR3 haplotype which is associated with autoimmune diseases and impaired functioning of the immune system.

HLA-A and HLA-B typing and immunohistochemical staining results of the 15 HNSCC lesions and 9 corresponding lymph node metastases are depicted in Table 3. Examples of positive, heterogeneous and negative immunohistochemical staining of tumor tissues and lymph node metastases are shown in Figure 1.

Tumor HN12 and HN15 were the only tumors with no detectable HLA loss, although HLA allele-specific expression could not be determined for all alleles. Total HLA-A and HLA-B expression-loss was detected in the primary tumors of HN04, HN10 and HN13. Immunohistochemical staining of these tumors with locus-specific mAbs HC-A2 (HLA-A) and HC-10 (HLA-B and HLA-C) confirmed total HLA-A, HLA-B and HLA-C expression-loss in HN10 and HN13. However, HC-A2 and HC-10 staining of HN04 was positive (data not shown). Positive HC-A2 staining may be caused by the cross-reactivity of HC-A2 with other HLA alleles than HLA-A, whereas positive HC-10 staining may be due to HLA-C expression in this tumor sample.

Seven out of the 15 HNSCC lesions (HN01, HN03, HN05, HN06, HN07, HN11 and HN14) showed HLA-B allele-specific loss in the primary lesions, of which two tumors (HN06 & HN14) had also HLA-A allele-specific loss. Summarized, HLA-B expression-loss was detected in 10 HNSCC lesions whereas only 5 tumors showed HLA-A expression-loss. None of the tumors had solely HLA-A expression-loss. These results suggest that HLA-B expression-loss is more frequent than HLA-A expression-loss in HNSCC lesions.

Combining HLA expression-loss data with haplotype analysis is helpful in defining the HLA loss phenotypes. HLA-A and HLA-B allele-specific loss of HN06 and HN14 can be defined as haplotype loss. The HLA-A*0301/B*0702 haplotype of HN07 appeared to be heterogeneously affected in the primary HNSCC lesion. Of the five tumor patients with the HLA-B8/DR3 haplotype, three (HN03, HN04 and HN11) showed expression-loss of the HLA-B8 antigen in the primary lesion as well as in the metastasis. HN06 showed a heterogeneous expression profile in the lymph node metastasis and HN08 had a heterogeneous expression profile in the primary lesion whereas the metastasis showed expression-loss. It can be concluded that the expression of the HLA-B*0801 allele is frequently affected in these HNSCC lesions and lymph node metastases.

HLA allele-specific expression in the metastasis completely corresponded with the expression in the primary lesion for 4 out of the 9 HNSCC (Table 3). However, five HNSCC lesions had a different HLA expression profile in the primary tumor

Table 3. Immunohistochemical staining results of HLA-A and HLA-B allelic product-specific human mAbs

	HLA-A & HLA-B alleles [a]				IH staining [b]				
Tumor	A1	A2	B1	B2		A1	A2	B1	B2
HN01	***A*030101***	***A*3201***	***B*5108***	***B*15010101***	Prim	+	n.a.	+	-
					Met	+	n.a.	+	-
HN02	***A*02***	***A*32***	***B*40***	***B*51***	Prim	+	n.a.	H	H
					Met	+	n.a.	H	H
HN03	***A*010101***	***A*010101***	***B*0801***	***B*3801***	Prim	H	H	-	n.a.
					Met	H	H	-	n.a.
HN04	***A*010101***	***A*02010101***	***B*0801***	***B*070201***	Prim	-	-	-	-
					Met	-	-	-	-
HN05	***A*02010101***	***A*02010101***	***B*510101***	***B*4002***	Prim	+	+	+	-
					Met	+	+	H	-
HN06	***A*010101***	***A*02010101***	***B*0801***	***B*070201***	Prim	H	-	+	-
					Met	H	-	H	-
HN07	***A*02010101***	***A*03010101***	***B*510101***	***B*070201***	Prim	+	H	-	H
					Met	+	+	-	H
HN08	***A*010101***	***A*02010101***	***B*0801***	***B*3701***	Prim	+	H	H	n.a.
					Met	+	+	-	n.a.
HN09	***A*02010101***	***A*24020101***	***B*070201***	***B*15010101***	Prim	+	+	H	H
					Met	+	+	H	+
HN10	***A*02010101***	***A*02010101***	***B*400102***	***B*510101***	Prim	-	-	-	-
HN11	***A*02010101***	***A*3201***	***B*4002***	***B*0801***	Prim	+	n.a.	+	-
HN12	A*010101	A*3301	B*1402	B*510101	Prim	+	n.a.	n.a.	+
HN13	***A*02010101***	***A*02010101***	***B*15010101***	***B*4001***	Prim	-	-	-	-
HN14	***A*010101***	***A*02010101***	***B*510101***	***B*15010101***	Prim	-	+	-	+
HN15	***A*010101***	***A*24020101***	***B*570101***	***B*510101***	Prim	n.a.	+	+	+

a) HLA-A and HLA-B alleles according to the defined haplotypes as A1/B1 and A2/B2 in bold italics. For HN02, only SSP typing could be performed

b) the HLA allele-specific immunohistochemical staining results of the primary tumor (Prim) and lymph node metastases (Met) as positive staining (+), heterogeneous staining (H), negative staining (-) and no mAb available (n.a.) for immunohistochemical staining

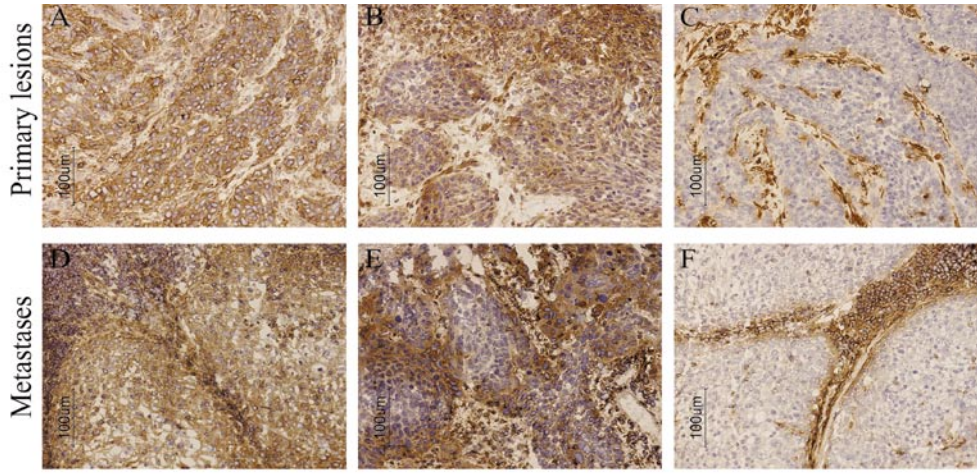

Figure 1. Immunohistochemical staining of primary HNSCC lesions and lymph node metastases with the human biotin-labeled mAbs showing a) positive staining of mAb IND2D12 (B15) in the primary lesion of HN14; b) heterogeneous staining of mAb VTM1F11 (B7) in the primary lesion of HN09; c) negative staining of mAb HDG8D9 (B51) in the primary lesion of HN14; d) positive staining of mAb SN607D8 (A2) in the lymph node metastasis of HN07; e) heterogeneous staining of mAb GV5D1 (A1) in the lymph node metastasis of HN06; f) negative staining of mAb HDG8D9 (B51) in the lymph node metastasis of HN07. Staining of stromal tissue and lymphocytes was used as a positive control in the negative tumors and lymph node metastasis, respectively.

compared to their lymph node metastasis. Tumor HN05 and HN06 showed positive expression in the primary lesion of the alleles HLA-B*510101 and HLA-B*0801 respectively, whereas their respective metastases had a heterogeneous expression pattern. A heterogeneous expression pattern for the HLA-A*0301, HLA-A*0201 and HLA-B*1501 alleles was seen in the primary lesions of HN07, HN08 and HN09 respectively, whereas the corresponding metastases showed positive expression. The primary lesion of tumor HN08 also had a heterogeneous expression profile for the HLA-B*0801 allele, whereas its metastasis showed expression-loss. Heterogeneous HLA expression was detected in primary tumors as well as in the corresponding lymph node metastases of HN02, HN03, HN06, HN07 and HN09.

From these data it can be concluded that: i) the majority of HNSCC had HLA allele-specific expression-loss; ii) HLA-B expression, and in particular expression of the HLA-B*0801 allele, was frequently affected; iii) HLA allele-specific heterogeneous expression was frequently detected in HNSCC; iv) HLA typing and haplotype analysis is useful in defining the HLA loss phenotypes; v) for the majority of HNSCC patients, the HLA expression profile differed between primary tumor and lymph node metastasis; vi) HLA allele-specific mAbs are required to adequately study HLA expression-loss in tumors and metastases.

6.4 Discussion

We analyzed HLA-A and HLA-B expression profiles in 15 HNSCC lesions and 9 corresponding lymph node metastases by immunohistochemical staining using allele-specific biotinylated human mAbs. Since the efficacy of T cell-based immunotherapy largely depends on the presentation of TAA by HLA class I antigens to cytotoxic T cells, it is of crucial importance to identify HLA expression-loss in tumors at the allele-specific level. Moreover, it increases and refines our knowledge of HLA expression-loss in tumor development.

Strikingly, five HNSCC patients have the HLA-B8/DR3 haplotype. This haplotype is well known for its association with a wide variety of diseases with autoimmune features in Caucasian populations [27]. Healthy individuals with the HLA-B8/DR3 haplotype have an impaired IL-2 production resulting in an affected lymphocyte activation [28]. IL-2 is a T helper 1 cytokine and is involved in the mediation of anti-tumor responses by the immune system. Moreover, activation-induced apoptosis of T cells is far lower in HLA-B8/DR3 positive compared to HLA-B8/DR3 negative individuals [29]. This affects the maintenance of immune homeostasis and selective T cell deletion. The 'overload' of T cells may not only be involved in autoimmune mediated diseases, but may also stimulate tumor cell eradication. Therefore, the high frequency of HLA-B8 expression-loss in the HNSCC lesions may illustrate a negative immune selection pressure against HLA-B8 positive tumor cells by the increased immune responsiveness in these individuals.

Based upon the results obtained from this study, our data indicated that HLA-B expression was more frequently lost than HLA-A expression. However, as we previously described, immunohistochemical staining of 102 HNSCC with mAb HC-A2 (HLA-A) and HC-10 (HLA-B & HLA-C) suggested that HLA-A expression was more frequently affected [21]. This discrepancy may be explained by the fact that mAb HC-10 is not specific for HLA-B. A high frequency of HLA-B expression-loss could be masked by HLA-C expression when using the cross reacting HC-10 mAb. In melanoma cell lines, it also has been reported that the frequency of HLA-B downregulation was higher compared to HLA-A [30]. Expanding the HNSCC panel for HLA allelic product-specific immunohistochemical staining will allow us to draw firmer conclusions.

Total HLA-A and HLA-B expression-loss was detected in three tumors (Table 3). Whether this was caused by HLA-A and HLA-B locus-loss or due to defects in HLA expression regulation is not known. HLA-A and HLA-B allele-specific loss was demonstrated in 7 out of 15 HNSCC lesions. Thus, without allelic product-specific mAbs, HLA expression-loss would have remained undetected in almost 50% of the tumors. This is of crucial importance for immunotherapy. T cell-based immunotherapy directed against a TAA which is not presented on the tumor cell surface due to allelic expression-loss is obviously not effective. Moreover, almost

50% of the tumors showed heterogeneous HLA expression of one or more alleles in the primary lesion and/or the lymph node metastases. T cell-based immunotherapy for these tumors should be directed against a TAA that is homogeneously presented by an HLA allele throughout the tumor as well as the metastases. Immunotherapy directed against a heterogeneously presented TAA may stimulate the development and expansion of 'escape variants' of the primary lesion and/or metastases. Therefore, immunotherapy should be tailored to each tumor patient based on the HLA allele-specific expression profile in the tumor as well as the metastases.

For nine HNSCC lesions, a corresponding lymph node metastasis was included in the study. The HLA-A and HLA-B allele-specific expression profiles were different between the primary lesion and the metastasis in five tumor patients (Table 3). This emphasizes the importance of studying HLA allele-specific expression in both primary tumor and metastases. In two tumors (HN05 & HN06), the primary lesion showed positive expression of an HLA allele, whereas the lymph node metastases had a heterogeneous expression profile. This may represent the early phase of expression-loss of the allele in the metastases. In the three other tumors, heterogeneous expression was detected in the primary tumor, while positive expression (HN07 and HN09) and even expression-loss of an HLA allele (HN08) was demonstrated in the corresponding metastasis. In the former cases, subclones of the heterogeneous primary tumor with positive expression of the HLA-A*0301 and HLA-B*1501 alleles respectively, may have metastasized and in the latter case, a subclone with expression of the HLA-A*0201 allele and expression-loss of B*0801. On the other hand, the subclone may also have been positive or heterogeneous for HLA-B*0801 allele expression at the onset of metastasizing and losing its expression during further progression. Nevertheless, it is remarkable that over 50% of the cases had a different HLA allele-specific expression profile in the primary lesion compared to their lymph node metastasis. In contrast, it has been demonstrated that there is no difference between primary HNSCC lesions and lymph node metastases for p53 mutations [31]. Mutations in p53 are considered to be an early event in HNSCC tumorigenesis, HLA expression-loss, on the other hand, a late event [32]. HLA expression alterations may therefore progress differentially between the primary lesion and the metastases. It has been suggested that selective pressure by the immune system or immunotherapy may contribute to the generation of lesions with HLA class I defects [9]. Differences in the immune micro-environment between the primary lesion and the metastases may therefore result in a differential HLA expression-loss profile.

In conclusion, the majority of HNSCC showed HLA allele-specific expression-loss, especially of HLA-B. Heterogeneous HLA expression was frequently detected. For the majority HNSCC patients, the HLA expression profile differed between primary tumor and lymph node metastasis. Therefore, HLA allele-specific antibodies are required to adequately study HLA expression-loss in HNSCC and their

metastases. The same probably holds for other types of cancer. A large panel of HLA allelic product-specific human mAb is available for further development and testing by immunohistochemistry. By combining HLA typing and immunohistochemical staining of fresh tumor tissue using HLA allelic product-specific mAb, one can unravel the role of HLA expression-loss in tumor development and improve the efficacy of immunotherapy.

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Chapter 7

TAP1 and TAP2 Allele Frequencies in a SNaPshot™: no Evidence for Allelic Prevalence in HNSCC Patients compared to Dutch Controls.

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Abstract: The heterodimeric TAP complex plays a key role in immune surveillance. Forming the portal between the cytoplasm and the endoplasmic reticulum, it enables cells to present antigenic peptides by HLA class I antigens to cytotoxic T cells. Hereby, infected and malignant cells can be eradicated. Since the nature of the peptide determines whether an immune response is evoked, peptide selective transport by TAP may influence immune surveillance. Currently, there are seven TAP1 and four TAP2 alleles known. Each may have its own effect on peptide transport. In this study, we investigated whether TAP1 and TAP2 alleles are associated with the development of head and neck squamous cell carcinoma (HNSCC). We developed a high-throughput SNaPshot™ assay to determine the frequencies of the TAP1 and TAP2 alleles in 79 Dutch control subjects and 94 HNSCC patients. Strikingly, all control and HNSCC samples contained a TAP1*0101 allele, with the exception of one tumor patient. The most frequent alleles were TAP1*0101 (88.2%) and TAP2*0101 (81.2%). No significant difference was found between control subjects and HNSCC patients. Here, we report the TAP1 and TAP2 allele frequencies in the Dutch population, the development of a high-throughput TAP typing technique and a new TAP1*0501 allele.

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7.1 Introduction

In tumor immunology, antigen processing and presentation are the focus of many studies. Understanding the nature of tumor specific antigens and the way they are processed for presentation by human leukocyte antigen (HLA) class I molecules will improve the development of immunotherapy and cancer vaccines. The human antigen-processing machinery (APM) consists of multiple components, each with specific functions ranging from cytoplasmatic protein breakdown to the presentation of the antigenic peptide by HLA class I antigens on the cell surface [1, 2]. The transport of these peptides from the cytoplasm into the lumen of the endoplasmic reticulum (ER) and loading onto the HLA class I molecules is an essential process for the presentation to cytotoxic T lymphocytes (CTL). The translocation of these peptides is facilitated by the transporter associated with antigen processing (TAP). Tapasin forms a bridge between TAP and the HLA class I molecule to facilitate peptide loading [3]. The stable HLA-peptide complexes are subsequently transported to the cell surface for immune surveillance by CTL. Not surprisingly, TAP has been studied in many human diseases [4]. In melanoma, small-lung carcinoma, high-grade primary breast carcinoma lesions and cervix carcinoma, TAP downregulation has been associated with tumor development [5-8]. Seliger *et al.* 1997 reviewed the role of TAP in cancer as "TAP off—tumors on" [9]. Moreover, downregulation of TAP in melanoma is significantly correlated with the development of metastases [10].

TAP is a heterodimeric ABC transporter protein complex with the TAP1 and TAP2 subunits combining in a head-head/tail-tail orientation to create a central pocket on the ER lumenal side. A heteromeric peptide-binding domain and two hydrophilic nucleotide-binding domains complete the TAP complex [11, 12]. TAP1 and TAP2 are not only functionally associated with HLA, but the genes are also located in the HLA class II region on chromosome 6p [13]. Moreover, the human TAP1 and PSMB9 genes are coordinately regulated from a shared bi-directional promoter [14]. Cytoplasmatic peptides derived from intra-cellular proteins bind to the peptide-binding domain and are subsequently translocated in an ATP-dependent manner through the pore in the ER membrane [15]. Since TAP can transport peptides with long side chains, the pore should be considerable flexible [16]. However, the optimal peptide size is between 8 and 13 aminoacids [17].

Immune surveillance is most effective when cells are able to present a wide and representative range of antigenic peptides with their HLA molecules. This increases the chance of an immune reaction when a cell is infected or becomes neoplastic. TAP should therefore not be highly selective in peptide transport. However, it has been described that human TAP molecules select peptides for transport mainly according to the residues in the C-terminal and the three N-terminal positions [18]. This facilitates optimal binding of the peptide in the HLA class I binding groove, since the anchor residues are located at the C- and N-terminus of the peptide. This

in contrast to HLA class II, were the peptide anchor residues are mainly located in the middle of the peptide. In rat, mouse and hamster, it has even been demonstrated that allelic differences in TAP modifies the repertoire of peptides that can be translocated into the ER [16, 19-21]. This may be beneficial for tumor development when specific tumor associated antigens can not be translocated into the ER due to peptide selectivity of certain TAP alleles. Obst *et al.* 1995 investigated whether TAP polymorphisms in mice and humans have functional consequences for transport of peptide sets variable at the C-terminal residues. They suggested that a major contribution of human TAP polymorphism to disease progression and autoimmunity is not very likely [22]. It should be noted that they used human lymphoblastoid cell lines expressing eight different TAP1 and TAP2 alleles. At this time, eleven TAP1 and TAP2 alleles are known and registered in the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/index.html). Moreover, peptide accumulation was measured in the plateau phase. It has been discussed that, by this approach, only dramatic differences in TAP transport efficiency between different peptide substrates may be detectable [23]. Daniel *et al.* 1997 reported that there was no significant functional effect of human TAP polymorphisms on peptide selection [24]. However, they found some minor differences in peptide transport, concerning a lower permissiveness of TAP1*0101/TAP2*0201 for longer peptides and a potentially higher efficiency of TAP1*0103 (with any TAP2 allele), which require confirmation in further studies. The drawback of this study is that they investigated only the most frequent TAP1 and TAP2 alleles (TAP1*0101, TAP1*0102, TAP1*0103, TAP2*0101 and TAP2*0201). Therefore, only six different TAP1 and TAP2 complexes could be generated while it is currently known that there are 28 possible combinations (seven TAP1 and four TAP2 alleles). Recently, the prevalence of the TAP2 gene codon 565 'A' allele (Thr) in rheumatoid arthritis has been described in Chinese patients in Taiwan [25]. Although the precise involvement of the APM in the development of rheumatoid arthritis is not known, functional consequences of allelic polymorphisms in human TAP can not be ruled out.

Many studies involving TAP and cancer focus on TAP downregulation or expression loss. In this study we investigated whether there is an association between TAP polymorphisms and the development of head and neck squamous cell carcinoma (HNSCC). Therefore, we included all currently known TAP1 and TAP2 alleles that are registered in the IMGT/HLA database. Considering the low frequency of certain TAP alleles, a large tumor and control panel are required for this association study. Therefore, we developed a high-throughput TAP typing SNaPshot™ technique to study 94 HNSCC and 79 control samples in a quick and easy way. Here, we also report the detection of a new TAP1 allele, TAP1*0501 (HWS10002372–AY523970)[1] encountered in this study.

[1] The name TAP1*0501 has been officially assigned by the WHO Nomenclature Committee in April 2004. This follows the agreed policy that, subject to the conditions stated in the most recent Nomenclature report [26], names will be assigned to new sequences as they are identified. Lists of such new names will be published in the following WHO Nomenclature report.

7.2 Materials and Methods

7.2.1 DNA Samples

High quality DNA was isolated out of peripheral blood lymphocytes (PBL) of 79 control subjects (blood transfusion donors) and 94 HNSCC patients according to the salting-out method [27]. The HNSCC panel consisted of 14 hypopharynx, 19 oropharynx, 32 larynx and 29 oral cavity carcinoma of which 52% were metastasizing. For the validation of the SNaPshot™ technique, we used 10 high quality DNA samples of a reference panel available in our laboratory.

7.2.2 TAP1 & TAP2 Allele Typing using the SNaPshot™ Approach

The six known TAP1 alleles, the newly discovered TAP1*0501 allele and the four TAP2 alleles were determined with the SNaPshot™ Multiplex kit (ABI Prism®), based upon nine polymorphic positions in TAP1 exons 2, 4, 10 and TAP2 exon 9 until 11. These polymorphisms are representative for all TAP alleles. For each sample, the exon regions were amplified by four PCR's as follows: 100 ng DNA, 1× PE Buffer II (Perkin Elmer, USA), 2 mM $MgCl_2$ (Perkin Elmer, USA), 0.8 mM dNTP's (Invitrogen, the Netherlands), 2 U AmpliTaq (Perkin Elmer, USA), 10 pmol forward primer, 10 pmol reverse primer and deionized water to a final volume of 50 µl. The following four PCR primer-sets were used: TAP1 exon 2 forward *5'gggcggggcat cctcatctctaa'3*, reverse *5'ggacagccccaaacacctct'3*; TAP1 exon 4 forward *5'caaga ccctaaatgctgaaactgc'3*, reverse *5'gctatgccccttggatgctaa'3*; TAP1 exon 10 forward *5'aaccccagtagtcttgcctttat'3*, reverse *5'ggtgtctttgcctcgtcttc'3* and TAP2 exon 9 until 11 forward *5'cctgccttgggggtttac'3*, reverse *5'ccaggcgggaatagaggt'3*. Amplification was performed in a Perkin Elmer 9600 (Applied Biosystems, the Netherlands) using the following program: a preheating step of 94°C for 20 s followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 90 s and finally an extension step of 72°C for 5 min. Of each PCR reaction, 15 µl was purified with 5 U SAP (Amersham, the Netherlands) and 1 U ExoI (Amersham, the Netherlands) for one hour at 37°C, followed by enzyme inactivation for 15 min at 75°C and subsequently pooled. The nine SNaPshot™ primers were combined at a final concentration of either 0.5 µM or 1 µM as shown in Table 1. For the SNaPshot™ reaction, 5 µl SNaPshot™ Multiplex Ready reaction Mix (Applied Biosystems, the Netherlands), 3 µl pooled purified PCR products, 1 µl SNaPshot™ primers and 1 µl deionized water were mixed. Thermal cycling was conducted on a Perkin Elmer 9600 (Applied Biosystems, the Netherlands) for 25 cycles of 96°C for 10 s, 65°C for 5 s and 60°C for 30 s. A post-extension treatment was performed by adding 1 U SAP for 1 hour at 37°C and deactivation of SAP at 75°C for 15 min. The samples were prepared for analysis on an ABI Prism 310 Genetic Analyzer by adding 9 µl HI-Di formamide (Applied Biosystems, the Netherlands) and 0.5 µl GeneScan™120 LIZ™ size standard (Applied Biosystems,

the Netherlands) to 0.5 µl purified SNaPshot™ product. After a brief vortex and a quick spin, the samples were denatured at 95°C for 5 min and placed on ice until ready to load. Analysis was performed according to the ABI Prism® SNaPshot™ Multiplex kit manual. TAP1 and TAP2 alleles were assigned to a sample based on the nine polymorphisms as depicted in Table 2.

Table 1. Overview of the TAP1 and TAP2 polymorphisms interrogated by the SNaPshot™ assay

Polymorphic position [a]	TAP exon [b]	5' poly-T [c]	5'-SNaPshot™ primers-3' [d]	Pool (µM) [e]
TAP1 p599	2	4	tggaatggccatctccc	0.5
TAP1 p997	4	13	agcagaggcagggtga	0.5
TAP1 p1910	10	23	ggctcccagcctcg	0.5
TAP1 p1943	10	29	caacgccactgcctgt	1.0
TAP1 p1983	10	31	catcatccaggataagtacaca	0.5
TAP1 p1927	10	70	gctgacccccctgaca	1.0
TAP2 p1693	9	40	ggttctgtgaggaacaacatt	1.0
TAP2 p1951	11	52	cactgtgcgatccccac	1.0
TAP2 p1993	11	63	gggcgctgaactg	1.0

a) location in the coding sequence

b) exon number

c) number of thymidines in the 5' poly-T tail of the SNaPshot™ primer

d) sequence of the SNaPshot™ primer

e) concentration in the SNaPshot™ primer pool

7.2.3 Validation of the TAP SNaPshot™

Of the 10 reference DNA samples, the four TAP1 and TAP2 exon region PCR products were sequenced to confirm the amplification of the correct genomic regions and to determine the TAP1 and TAP2 alleles. These samples were used as control samples to validate the newly developed SNaPshot™ technique. In one sample, we discovered a new polymorphism in exon 10 of the TAP1 gene by sequencing. The complete mRNA sequence of this sample was amplified using the Long Extension PCR kit (Roche, Almere, the Netherlands) and cloned in a pCR®2.1 vector (Invitrogen, the Netherlands) by TA cloning. The cDNA sequence of both alleles were determined by bi-directional sequencing of three clones per allele. The sequence of the new allele was submitted to the WHO Nomenclature Committee and the name TAP1*0501 was officially assigned. Since TAP1 exon 10 codes for a part of the cytoplasmatic located peptide-binding pocket of the TAP complex, polymorphisms in this exon are of particular interest. Therefore, we also sequenced exon 10 of all 79 control DNA samples, but no additional new polymorphisms were found.

Table 2. Overview of the TAP1 and TAP2 alleles

	TAP1						TAP2		
	p599 exon 2	p997 exon 4	p1910 exon 10	p1943 exon 10	p1983 exon 10	p1927 exon 10	p1693 exon 9	p1951 exon 11	p1993 exon 11
TAP1*0101	G	A	A	G	G	G	-	-	-
TAP1*0102N	A	A	A	G	G	G	-	-	-
TAP1*020101	G	G	G	G	G	G	-	-	-
TAP1*020102	G	G	G	G	A	G	-	-	-
TAP1*0301	G	G	A	G	G	G	-	-	-
TAP1*0401	G	G	G	A	G	G	-	-	-
TAP1*0501	G	G	G	G	G	T	-	-	-
TAP2*0101	-	-	-	-	-	-	G	C	A
TAP2*0102	-	-	-	-	-	-	A	C	A
TAP2*0103	-	-	-	-	-	-	G	T	A
TAP2*0201	-	-	-	-	-	-	G	C	G

For each allele, the specific nucleotides at the polymorphic positions interrogated by the SNaPshot™ assay are indicated. Using this overview, the SNaPshot™ profile of a DNA sample can easily be analyzed. The order of the peaks obtained in the SNaPshot™ profile corresponds with the polymorphic positions in the Table, except for p1927 which forms the last peak.

7.2.4 Statistics

The allelic frequency was expressed as a percentage of the total number of alleles. Results from the control subjects and HNSCC patients were compared with a χ^2 test for statistical significance. When the assumption of the χ^2 test was violated, i.e. one cell had an expected count < 1 or more than 20% of the cells had an expected count of < 5, Fisher's exact test was used. A p value less than 0.05 was considered statistically significant. The χ^2 test was also used to determine whether there was a significant difference between metastasizing HNSCC, non-metastasizing HNSCC, hypopharynx, oropharynx, larynx or oral cavity carcinoma and control subjects.

7.3 Results

*7.3.1 The New TAP1*0501 Allele*

By sequencing TAP1 exon 10 of a reference sample for the validation of the SNaPshot™ technique, we found a new TAP1 allele which has been officially named TAP1*0501 by the WHO Nomenclature Committee in April 2004 [26]. The coding sequence is depicted in Figure 1. The coding sequence of the TAP1*0501 allele contains 10 polymorphisms compared to TAP1*0101, shown in bold, of which seven

are unique, shown in lower case, compared to all other known TAP1 alleles. This emphasizes the importance of sequencing the complete coding sequence when a new TAP allele is found. Compared to TAP1*0101, these polymorphisms give rise to five amino acid changes, shown by the boxed codons, of which three are unique amongst all currently known TAP1 alleles. The codons of these unique aminoacid changes are shown by the boxed codons containing a lower case polymorphism. The alignment of the TAP1*0501 coding sequence with all other TAP1 alleles can be viewed at the IMGT/HLA sequence database (http://www.ebi.ac.uk/imgt/hla/align.html) [28].

Table 3. Allelic frequencies of the TAP1 and TAP2 alleles

	HNSCC [a] (n=94)	Controls [b] (n=79)	Total [c] (n=173)
TAP1*0101	165 (87.8%)	140 (88.6%)	305 (88.2%)
TAP1*0102N	0 (0.0%)	0 (0.0%)	0 (0.0%)
TAP1*020101	16 (8.5%)	12 (7.6%)	28 (8.1%)
TAP1*020102	3 (1.6%)	4 (2.5%)	7 (2.0%)
TAP1*0301	4 (2.1%)	2 (1.3%)	6 (1.7%)
TAP1*0401	0 (0.0%)	0 (0.0%)	0 (0.0%)
TAP1*0501	0 (0.0%)	0 (0.0%)	0 (0.0%)
TAP2*0101	146 (77.7%)	135 (85.4%)	281 (81.2%)
TAP2*0102	16 (8.5%)	8 (5.1%)	24 (6.9%)
TAP2*0103	22 (11.7%)	15 (9.5%)	37 (10.7%)
TAP2*0201	4 (2.1%)	0 (0.0%)	4 (1.2%)

The absolute number of alleles is given, followed by the allelic frequency in percentages between brackets.

a) in the HNSCC patients

b) in the Dutch control subjects

c) in the HNSCC patients and Dutch control subjects combined

7.3.2 TAP1 & TAP2 Allele Frequencies in HNSCC and Control

The 79 control subjects and 94 HNSCC patients were typed for TAP1 and TAP2 using the SNaPshot™ technique. In Figure 2, an example of the TAP SNaPshot™ profile is depicted of a HNSCC patient which was typed as a TAP1*0101 and TAP2*0101,0103. The heterozygous TAP2 typing is clearly indicated by the heterozygous G/A peak. The allelic and phenotype frequencies of the control subjects and HNSCC patients are shown in Tables 3 and 4, respectively. The expected frequencies of the TAP phenotypes shown in Table 4[d] were calculated from the total allelic frequencies depicted in Table 3[c]. There was no significant difference in the allelic and phenotype frequencies of the TAP1 and TAP2 alleles between control

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   1  ATGGCTAGCTCTAGGTGTCCCGCTCCCCGCGGGTGCCGCTGCCTCCCCGG
  51  AGCTTCTCTCGCATGGCTGGGGACAGTACTGCTACTTCTCGCCGACTGGG
 101  TGCTGCTCCGGACCGCGCTGCCCCGCATATTCTCCCTGCTGGTGCCCACC
 151  GCGCTGCCACTGCTCCGGGTCTGGGCGGTGGGCCTGAGCCGCTGGGCCGT
 201  GCTCTGGCTGGGGGCCTGCGGGGTCCTCAGGGCAACGGTTGGCTCCAAGA
 251  GCGAAAACGCAGGTGCCCAGGGCTGGCTGGCTGCTTTGAAGCCATTAGCT
 301  GCGGCACTGGGCTTGGCCCTGCCGGGACTTGCCTTGTTCCGAGAGCTGAT
 351  CTCATGGGGAGCCCCCGGGTCCGCGGATAGCACCAGGCTACcgGCACTGGG
 401  GAAGTCACCCTACCGCCTTCGTcGTCAGTTATGCAGCGGCACTGCCCGCA
 451  GCAGCCCTGTGGCACAAACTCGGGAGCCTCTGGGTGCCCGGCGGTCAGGG
 501  CGGCTCTGGAAACCCTGTGCGTCGGCTTCTAGGCTGCCTGGGCTCGGAGA
 551  CGCGCCGCCTCTCGCTGTTCCTGGTCCTGGTGGTCCTCTCCTCTCTTGGG
 601  GAGATGGCCATTCCATTCTTTACGGGCCGCCTCACTGACTGGATTCTACA
 651  AGATGGCTCAGCCGATACCTTCACTCGAAACTTAACTCTCATGTCCATTC
 701  TCACCATAGCCAGTGCAGTGCTGGAGTTCGTGGGTGACGGGATCTATAAC
 751  AACACCATGGGtCACGTGCACAGCCACTTGCAGGGAGAGGTGTTTGGGGC
 801  TGTCCTGCGCCAGGAGACGGAGTTTTTCCAACAGAACCAGACAGGTAACA
 851  TCATGTCTCGGGTAACAGAGGACACGTCCACCCTGAGTGATTCTCTGAGT
 901  GAGAATCTGAGCTTATTTCTGTGGTACCTGGTGCGAGGCCTATGTCTCTT
 951  GGGGATCATGCTCTGGGGATCAGTGTCCCTCACCATGGTCACCCTGGTCA
1001  CCCTGCCTCTGCTTTTCCTTCTGCCCAAGAAGGTGGGAAAATGGTACCAG
1051  TTGCTGGAAGTGCAGGTGCGGGAATCTCTGGCAAAGTCCAGCCAGGTGGC
1101  CATTGAGGtcCTGTCGGCCATGCCTACAGTTCGAAGCTTTGCCAACGAGG
1151  AGGGCGAAGCCCAGAAGTTTAGGGAAAAGCTGCAAGAAATAAAGACACTC
1201  AACCAGAAGGAGGCTGTGGCCTATGCAGTCAACTCCTGGACCACTAGTAT
1251  TTCAGGTATGCTGCTGAAAGTGGGAATCCTCTACATTGGTGGGCAGCTGG
1301  TGACCAGTGGGGCTGTAAGCAGTGGGAACCTTGTCACATTTGTTCTCTAC
1351  CAGATGCAGTTCACCCAGGCTGTGGAGGTACTGCTCTCCATCTACCCCAG
1401  AGTACAGAAGGCTGTGGGCTCCTCAGAGAAAATATTTGAGTACCTGGACC
1451  GCACCCCTCGCTGCCCACCCAGTGGTCTGTTGACTCCCTTACACTTGGAG
1501  GGCCTTGTCCAGTTCCAAGATGTCTCCTTTGCCTACCCAAACCGCCCAGA
1551  TaTCTTAGTGCTACAGGGGCTGACATTCACCCTACGCCCTGGCGAGGTGA
1601  CGGCGCTGGTGGGACCCAATGGGTCTGGGAAGAGCACAGTGGCTGCCCTG
1651  CTGCAGAATCTGTACCAGCCCACCGGGGGACAGCTGCTGTTGGATGGGAA
1701  GCCCCTTCCCCAATATGAGCACCGCTACCTGCACAGGCAGGTGGCTGCAG
1751  TGGGACAAGAGCCACAGGTATTTGGAAGAAGTCTTCAAGAAAATATTGCC
1801  TATGGCCTGACCCAGAAGCCAACTATGGAGGAAATCACAGCTGCTGCAGT
1851  AAAGTCTGGGGCCCATAGTTTCATCTCTGGACTCCCTCAGGGCTATGACA
1901  CAGAGGTAGGCGAGGCTGGGAGCCAGtTGTCAGGGGGTCAGCGACAGGCA
1951  GTGGCGTTGGCCCGAGCATTGATCCGGAAACCATGTGTACTTATCCTGGA
2001  TGATGCCACCAGTGCCCTGGATGCAAACAGCCAGTTACAGGTGGAGCAGC
2051  TCCTGTACGAAAGCCCTGAGCGGTACTCCCGCTCAGTGCTTCTCATCACC
2101  CAGCACCTCAGCCTGGTGGAGCAGGCTGACCACATCCTCTTTCTGGAAGG
2151  AGGCGCTATCCGGGAGGGGGGAACCCACCAGCAGCTCATGGAGAAAAAGG
2201  GGTGCTACTGGGCCATGGTGCAGGCTCCTGCAGATGCTCCAGAATGAAAG
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Figure 1. Coding sequence of the TAP1*0501 allele (AY523970) with the 10 polymorphisms compared to the TAP1*0101 alleles indicated by bold nucleotides. The seven unique polymorphisms that are not shared by any other TAP1 allele are depicted in lower case. The codon containing the polymorph position is indicated by underlined triplets for synonymous and boxed triplets for non-synonymous amino acids compared to TAP1*0101. Combining the unique, lower case, polymorphisms and the non-synonymous, boxed, codons shows that TAP1*0501 contains three unique amino acids compared to all TAP1 alleles.

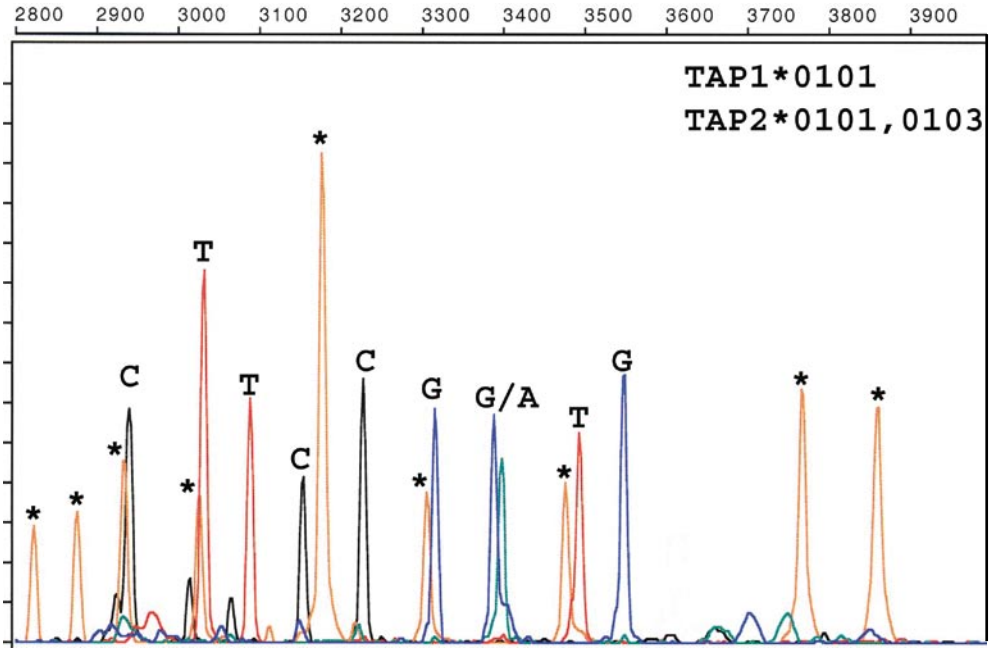

Figure 2. An example of the TAP SNaPshot™ profile of a HNSCC patient which was typed as a TAP1*0101 and TAP2*0101,0103. The nine interrogated polymorphisms are depicted by the colored peaks as follows: black, C; red, T; blue, G and green, A. The orange 120 LIZ™ size standard peaks are indicated by an asterisk. The first five and the last peaks indicate the TAP1 typing and peak numbers six until eight the TAP2 typing. Using Table 2, the TAP alleles can easily be assigned.

subjects and HNSCC patients. Of the 94 HNSCC, 73 (77.7%) were homozygous and 21 samples were heterozygous for TAP1. For TAP2, 72 (76.6%) tumor samples were homozygous and 22 patients were heterozygous. In de control samples, 61 (77.2%) were homozygous and 18 heterozygous for TAP1, whereas for TAP2, 68 (86%) samples were homozygous and 11 heterozygous. The most frequent alleles are the TAP1*0101 and the TAP2*0101. Strikingly, all control and HNSCC samples contained at least one TAP1*0101 allele with the exception of one tumor sample. This one was homozygous for TAP1*020101. Although TAP2*0101 was the most frequent allele in both tumors and control, nine, five and two samples were homozygous for TAP2*0102, TAP2*0103 and TAP2*0201, respectively. All other samples contained at least one TAP2*0101 allele.

We also analyzed the allelic frequency differences between metastasizing HNSCC, non-metastasizing HNSCC, hypopharynx, oropharynx, larynx or oral cavity carcinoma and control subjects. No statistical differences were found.

Table 4. Phenotype frequencies of TAP1 and TAP2

Allele 1	Allele 2	HNSCC[a] (n=94)	Controls[b] (n=79)	Total[c] (n=173)	Expected Frequency[d]
TAP1*0101	-	72 (76.6%)	61 (77.2%)	133 (76.9%)	77.8%
TAP1*020101	-	1 (1.0%)	0 (0.0%)	1 (0.6%)	0.7%
TAP1*0101	TAP1*020101	14 (14.9%)	12 (15.2%)	26 (15.0%)	7.1%
TAP1*0101	TAP1*020102	3 (3.2%)	4 (5.1%)	7 (4.0%)	1.7%
TAP1*0101	TAP1*0301	4 (4.3%)	2 (2.5%)	6 (3.5%)	1.5%
TAP2*0101	-	62 (66.0%)	62 (78.5%)	124 (71.7%)	65.9%
TAP2*0102	-	6 (6.4%)	3 (3.8%)	9 (5.2%)	0.5%
TAP2*0103	-	2 (2.1%)	3 (3.8%)	5 (2.9%)	1.1%
TAP2*0201	-	2 (2.1%)	0 (0.0%)	2 (1.2%)	0.01%
TAP2*0101	TAP2*0102	4 (4.3%)	2 (2.5%)	6 (3.4%)	5.6%
TAP2*0101	TAP2*0103	18 (19.1%)	9 (11.4%)	27 (15.6%)	8.7%

The absolute number of each phenotype is given, followed by the phenotype frequency in percentages between brackets.

a) in HNSCC patients

b) in Dutch control subjects

c) in HNSCC patients and Dutch control subjects combined

d) expected frequencies of the TAP phenotypes were calculated from the total allelic frequencies shown in Table 3[c]

7.4 Discussion

Considering the importance of TAP in antigen processing and presentation, the TAP1 and TAP2 genes have been the focus of many studies. Here, we studied whether TAP1 and TAP2 alleles are associated with the development of HNSCC. In this study, the new TAP1*0501 allele was identified. This allele is quite distinct from the other TAP1 alleles. It contains 10 nucleotide polymorphisms compared to TAP1*0101 of which three occur in the other TAP1 alleles and seven are unique. On the amino acid level, there are five amino acid changes compared to TAP1*0101 of which two are shared with other TAP1 alleles and three are unique. These three non-synonymous amino acids are located in exon one, five and seven. In particular, the proline in exon 1 of TAP1*0501 can influence its protein structure [29]. What the effect of these unique amino acids is on the formation and the function of the heteromeric TAP1 and TAP2 complex is not known. The TAP1*0501 allele has a low allelic frequency in Dutch Caucasians and was encountered once in 183 samples (79 control subjects,

94 HNSCC patients and 10 reference panel samples). However, considering the significant variance in allelic frequency of the TAP1*0401 allele between different populations (e.g. Africans, Europeans and Americans) [30], it is interesting to study the allelic frequency of this new allele in other ethnic groups.

It should be noted that the coding sequence of TAP1 reported in X57522 is not concordant with X66401 [31, 32]. Exon 1 starts 180 bp downstream at position 211 in this sequence. This is in accordance with the coding sequence of TAP1 as reported in the IMGT/HLA database.

We applied the SNaPshot™ technique to determine the TAP1 and TAP2 alleles of 79 Dutch control subjects and 94 HNSCC patients in a high-throughput manner. This is an important advantage since the majority of the TAP alleles are low frequency alleles. Large sample panels are required to be able to detect them and to conduct a thorough association study. A disadvantage of the SNaPshot™ technique, in contrast to sequencing, is that one can not detect new alleles with polymorphisms at other locations than interrogated. By validating the SNaPshot™ technique by simultaneous DNA sequencing of TAP1 exon 10, we detected one new allele (TAP*0501) in 89 samples (79 control subjects and 10 reference panel samples). This new allele was only found by SNaPshot™ analysis in a reference panel sample and not in Dutch control subjects (n=79) and HNSCC patients (n=94). Considering the low frequency of lately discovered TAP1 and TAP2 alleles, new alleles are not likely to be frequently found. However, using the high-throughput property of the SNaPshot™ technique, the search for new TAP alleles can be optimized. Instead of labor-intensive bi-directional sequencing of large DNA sample panels, the SNaPshot™ technique can be easily applied to screen panels for homozygous samples with low frequency TAP alleles. When such samples are more frequently observed than expected, based on statistics, they may contain new alleles. Besides, new polymorphisms at the interrogated positions of the SNaPshot™ assay are also detected. For three homozygous phenotypes, TAP1*0101, TAP1*020101 and TAP2*0101, the observed frequency in our HNSCC and control panel is in concordance with the expected frequency. However, for the homozygous TAP2*0102, TAP2*0103 and TAP2*0201 phenotypes, the observed frequencies (5.2%, 2.9% and 1.2%, respectively) are higher than the expected frequencies (0.5%, 1.1% and 0.01%, respectively). Especially, the homozygous TAP2*0102 and TAP2*0201 samples are of interest to identify new alleles. We are currently sequencing these samples to confirm whether they are homozygous or contain a new allele. Although this strategy may miss some new alleles in e.g. homozygous samples with high frequency alleles, it saves a lot of effort and time.

In our Dutch control subjects and HNSCC patients panels, TAP1*0102N, TAP1*0401 and the newly discovered TAP1*0501 are the only alleles that were not found. TAP1*0102N differs only from TAP1*0101 by a deletion at position 599

which results in a premature stop codon. Therefore, TAP1*0102N is often included in the TAP1*0101 analysis and not much is known about its frequency. The allelic frequency of TAP1*0401 is approximately 1.2% in European Caucasians. This is comparable with our observed frequency. In American Caucasians, it varies between 3.7% and 5.8% while in Africans it is approximately 25% [33-35]. The allelic frequencies of the TAP1 alleles match best with those in the French and American Caucasian population and differ not significantly from the allelic frequencies in the German population [33-35]. All four known TAP2 alleles were identified by the SNaPshot™. The observed frequencies in our Dutch control and tumor panel are quite distinct compared to French Caucasians and other populations. Compared to the French, the Dutch TAP2*0101 allelic frequency is almost twice as much. Moreover, TAP2*0201 occurs 20 times more frequent in the French population than in our Dutch control and tumor panel. Also between groups of other ethnic origin (e.g. Indians and Africans), the allelic frequencies of TAP2 differ [36]. It can be concluded that there is more variation in the allelic frequency distribution between different populations for TAP2 alleles than for TAP1 alleles. It should be noted that the TAP2*0202 allele, which is restricted to black African populations, is not officially registered at the IMGT/HLA sequence database [36]. It is therefore not included in the SNaPshot™. Moreover, in several recent papers, the TAP nomenclature as assigned by the WHO Nomenclature Committee is not succeeded. This hinders a fair comparison of the allele data. In our approach, we followed the guideline of the TAP nomenclature as assigned by the WHO Nomenclature Committee [26].

There was no statistical difference in the TAP1 and TAP2 allele distribution between HNSCC patients and control subjects, as well as between metastasizing HNSCC, non-metastasizing HNSCC, hypopharynx, oropharynx, larynx or oral cavity carcinoma and control subjects. It can therefore be concluded that there is no association between TAP alleles and the development of HNSCC or metastasis. However, it has already been shown that loss of TAP expression and mutations in the TAP genes do play a role in tumor development [9, 37]. The fact that TAP1 is strongly induced by p53 and DNA-damaging agents through a p53-responsive element only emphasizes the role of TAP expression, rather than polymorphisms, in tumor surveillance. Studying the gene expression levels of TAP expression and other components of the human antigen-processing machinery will improve our understanding of antigen processing and presentation in tumor cells.

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Chapter 8

Gene Expression Profile of HLA Class I Antigen Processing Genes in Head and Neck Squamous Cell Carcinoma.

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Abstract: Antigen processing is essential for immune surveillance. Tumor cells presenting antigenic peptides by HLA class I molecules can be recognized and eradicated by immune cells. Defects in the antigen-processing machinery (APM) affect HLA class I expression and immune surveillance. Therefore, we studied the gene expression of 17 APM genes and IFN-γ in 23 HNSCC by quantitative PCR in comparison with the expression in tumor-adjacent, healthy control tissue. We found a significant upregulation of PSMB7 *(p = 0.002)*, PSMB8 *(p = 0.044)*, PSMB9 *(p = 0.004)*, TAP1 *(p = 0.002)* and tapasin *(p = 0.002)*, whereas TPPII gene expression was significantly downregulated *(p = 0.004)*. The mean expression levels of PSMB9, PSMB10 and TAP2 were considerable lower compared to their functional equivalents in tumor as well as control. For the other genes tested, the gene expression profile of the 23 HNSCC patients was approximately the same or higher in the tumor tissue compared to the control tissue. Our results indicate that there are no gene defects that affect the APM and IFN-γ gene expression at the mRNA level in HNSCC and support the theory that downregulation or expression loss of APM genes is likely to be caused by regulating factors.

Submitted

8.1 Introduction

More and more studies demonstrate that immune cells do not solely fight infections, but also play an important role in the control of malignancies [1-3]. Presentation of antigenic peptides by HLA class I molecules enables the adaptive immune system to monitor and respond to invading pathogens and malignant transformation. Not surprisingly, HLA class I antigen expression in tumors has been the focus of many studies in which abnormalities in the HLA class I antigens were frequently found [4, 5]. HLA cell surface expression is tightly regulated and requires a complex machinery, also known as the antigen-processing machinery (APM) [6].

HLA class I antigen processing involves three main steps: cytosolic peptide generation by protein degradation, peptide transport into the endoplasmic reticulum (ER) and peptide assembly with HLA class I molecules. The details of this complex mechanism have been thoroughly reviewed [7-9]. In short, endogenous proteins, or tumor-associated antigens (TAA) in tumor cells, are degraded by the proteasome [10]. This is a large multi-catalytic protease complex of which specific subunits (i.e. PSMB5, PSMB6 and PSMB7) determine the efficacy to produce antigenic peptides capable of binding HLA class I antigens. In the immunoproteasome, these specific subunits are exchanged by PSMB8, PSMB9 and PSMB10, which are the functional equivalents of PSMB5, PSMB6 and PSMB7, respectively [11, 12]. The immunoproteasome generates peptides with an enhanced fit for HLA class I antigens [13]. The proteasome activator PA28 protein can enhance the peptide generation by the core proteasome by facilitating dual cleavage [14]. Recently, it has been shown that the novel C-terminal cleaving protease tri-peptidyl peptidase II (TPPII) is also involved in antigen processing [13, 15-17]. It is even suggested that TPPII can substitute proteasome functions [18, 19]. After C-terminal cleavage by the proteasome and TPPII, the generated oligopeptides are processed at the N-terminus by aminopeptidases such as leucine aminopeptidase (LAP) and thimet oligopeptidase (TOP) [20]. These proteolytic processes, required for antigen presentation, form a delicate balance between generating and destroying epitopes [21]. From the cytosol, the antigenic peptides are translocated into the ER by the heterodimeric transporter associated with antigen processing (TAP) which consist of a TAP1 and a TAP2 protein [22]. In the ER, the antigenic precursor peptides are trimmed by ER aminopeptidase 1 (ERAP1) to optimally fit the HLA class I molecules [23, 24]. Chaperone proteins in the ER (i.e. calreticulin, calnexin, tapasin and ERp57) facilitate the assembly of the HLA heavy chain, the β2 microglobulin light chain and the antigenic peptide for presentation on the cell surface [7, 9, 25].

Defects in the components of the APM affect immune responsiveness and stimulate tumor growth by downregulation of HLA class I antigen cell surface expression [26, 27]. Several studies have shown that defects in the APM of tumor cells are frequently caused by regulatory abnormalities rather than structural gene defects

[28-30]. For example, downregulation of TAP1, PSMB8, PSMB9 and PSMB10 subunits has, amongst others, been found in tumor cell lines, primary melanoma and renal cell carcinoma [31-33]. The importance of TAP expression has even been reviewed as "TAP off – tumors on" [34]. Overexpression, on the other hand, of TOP and ERAP1 reduces the availability of peptides for HLA class I presentation by total degradation of oligopeptides in the cytosol and ER, respectively [35, 36].

In head and neck squamous cell carcinoma (HNSCC), loss of HLA expression is frequently observed [37, 38]. Peptide loading into the HLA-peptide binding groove is required for HLA antigen cell surface expression. The majority of these peptides are specifically generated by the APM. To investigate whether defects in the gene expression of APM proteins are involved in the development of HNSCC, we studied the gene expression of the 17 APM proteins in freshly frozen tumor tissue of 23 HNSCC patients. We included IFN-γ in our study since this immune-regulating cytokine is involved in the transcription regulation in the majority of the APM genes.

8.2 Materials and Methods

8.2.1 Tissue Samples

Freshly frozen tumor tissue and tumor-adjacent healthy tissue were obtained from 23 HNSCC patients that were surgically treated for this tumor at the departments of craniomaxillofacial surgery and otorhinolaryngology at the University Medical Centre Utrecht between 1996 and 2001. Of the 23 HNSCC patients, 15 were diagnosed with a metastasizing tumor. The tissue samples were selected, based on the tumor percentage of the tumor tissue section. A tumor percentage of at least 70% was required to exclude the need for microdissection. Adjacent healthy tissue was used as control tissue. Four 10 µm cryostat sections of the frozen tumor tissue and the healthy control tissue were cut for mRNA isolation.

8.2.2 Tissue Morphology

To visualize the tissue morphology, to assess the tumor percentage and to control for infiltrating cells, cryostat sections of the tumor and healthy control tissue were stained with heamatoxylin-eosin as follows. Cryostat tissue section of 6 µm were dried at RT for 10 min, fixed with 4% formaldehyde, phosphate-buffered (40 mmol/l) pH 7.0 (Klinipath, Duiven, the Netherlands) for 10 min, rinsed with demineralized water for 2 min and stained with heamatoxylin for 5 min. The sections were rinsed in running tap water for 10 min, stained with eosin for 30 sec, rinsed with demineralized water and dehydrated in an increasing ethanol-series. Finally, the sections were covered in xylene and mounted.

8.2.3 Cell Lines

An EBV transformed human B-cell lymphoblastoid cell line, BSM (IHW9032), was grown in RPMI 1640 medium (GIBCO™, Invitrogen, Breda) containing 10% v/v heat inactivated FCS and 1% v/v penicillin/streptomycin at 37°C in a 5% CO_2 incubator (Brouwer Engineering, de Meern). APM gene expression was stimulated by adding 200 U IFN-γ (Sigma-Aldrich Chemie BV, Zwijndrecht) per ml medium 48 hours before harvesting. RNA was isolated and used in the quantitative PCR as a positive control.

8.2.4 RNA Isolation and cDNA Synthesis

GenoPrep™ mRNA magnetic beads (GenoVision, Vienna) were used to isolate mRNA out of four 10 µm frozen tumor and control tissue sections in a final volume of 18 µl according to the manufacturer's manual. The magnetic beads are coated with, covalently bound, oligo(dT) nucleotides to which mRNA binds with its poly(A)-tail. Using a magnetic field, the beads and the mRNA can easily be extracted from the suspension.

Using Trizol® (GIBCO BRL®, Life Technologies, Breda), total RNA was isolated out of approximately 8×10^7 cells from the cell line BSM according to the manufacturer's manual. The yield of RNA obtained, was measured with a spectrophotometer at 260/280 nm (Perkin Elmer).

For cDNA synthesis, 18 µl tissue sample mRNA or 6 µg BSM cell line total RNA in 28 µl deionized water were incubated with 1 µl oligo$(dT)_{15}$ primers 0.5 µg/µl (Promega, Leiden) and 1 µl random primers 0.5 µg/µl (Promega, Leiden) at 70°C for 5 min. Subsequently, the solution was chilled on ice and 12 µl RT-first strand buffer 5× (Invitrogen, Breda), 6 µl DTT 0.1 M (Promega, Leiden), 4 µl dNTP's 10 mM (Invitrogen, Breda), 1 µl RNAsin 40 U/µl (Invitrogen, Breda), 1 µl Superscript™ $RNaseH^-$ Reverse Transcriptase 200 U/µl (Invitrogen, Breda) and deionized water were added to a final volume of 60 µl. cDNA was synthesized at 42°C for one hour and stored at -20°C until further use.

8.2.5 Quantitative PCR

To study the gene expression at the RNA level of the 17 APM genes of interest, IFN-γ and the housekeeping genes Gusβ and TBP, Assay on Demand™ Gene expression reagents (Applera, Nieuwerkerk a/d IJssel) were used. For each gene, a quantitative PCR was performed in duplo for all 23 HNSCC and 23 corresponding control tissue samples in a 96-well optical plate with optical caps (ABI Prism™, Foster City, CA). BSM cell line derived cDNA and deionized water were included in duplo as positive and negative control, respectively. Each well contained 1.25 µl of the gene specific Assay on Demand™ Gene expression mix 20× (Applera, Nieuwerkerk a/d IJssel), 12.5 µl TaqMan® Universal PCR Master Mix 2×, product number 4326614 (Applera,

Nieuwerkerk a/d IJssel), 5 µl cDNA and deionized water to a final volume of 25 µl. The quantitative PCR was conducted in a ABI Prism™ 7700 Sequence Detector (Applied Biosystems, Nieuwerkerk a/d IJssel) using the following program: 10 min at 94°C followed by 45 cycles of 15 sec at 95°C and 1 min at 60° C. Mean Ct values of the duplo samples were calculated and the –ΔCt method: "– (Ct gene of interest – Ct housekeeping gene)", was used to determine the relative gene expression in tumor tissue and in healthy control tissue. For the analysis, we choose Gusβ as primary housekeeping gene, since it gave the least variation itself among the tumors and is described to be the most consistently expressed housekeeping gene in various tumor tissue types [39].

8.2.6 Data and Statistical Analysis

The paired T test was used to determine whether there was a statistical significant difference in gene expression between HNSCC samples and healthy controls ($p < 0.05$). We used the parametric distributed –ΔCt values for statistical analysis, instead of the non-parametric distributed relative expression expressed as $2^{-\Delta Ct}$. The obtained p values were corrected for the number (19) of gene parameters considered. The –ΔCt values relative to Gusβ are arbitrary and are only informative for relative differences between tumor and control tissue, and between genes.

8.3 Results

The gene expression in tumor tissue and healthy control tissue, directly adjacent to the tumor, of the 23 HNSCC patients was calculated relative to the Gusβ expression using the –ΔCt method: "– (Ct gene of interest – Ct housekeeping gene)". The mean –ΔCt value of all 23 patients for the 17 APM genes and IFN-γ is depicted in Figure 1. This Figure displays an overview in which the genes are clustered according to their function. Details of individual gene expression are described below.

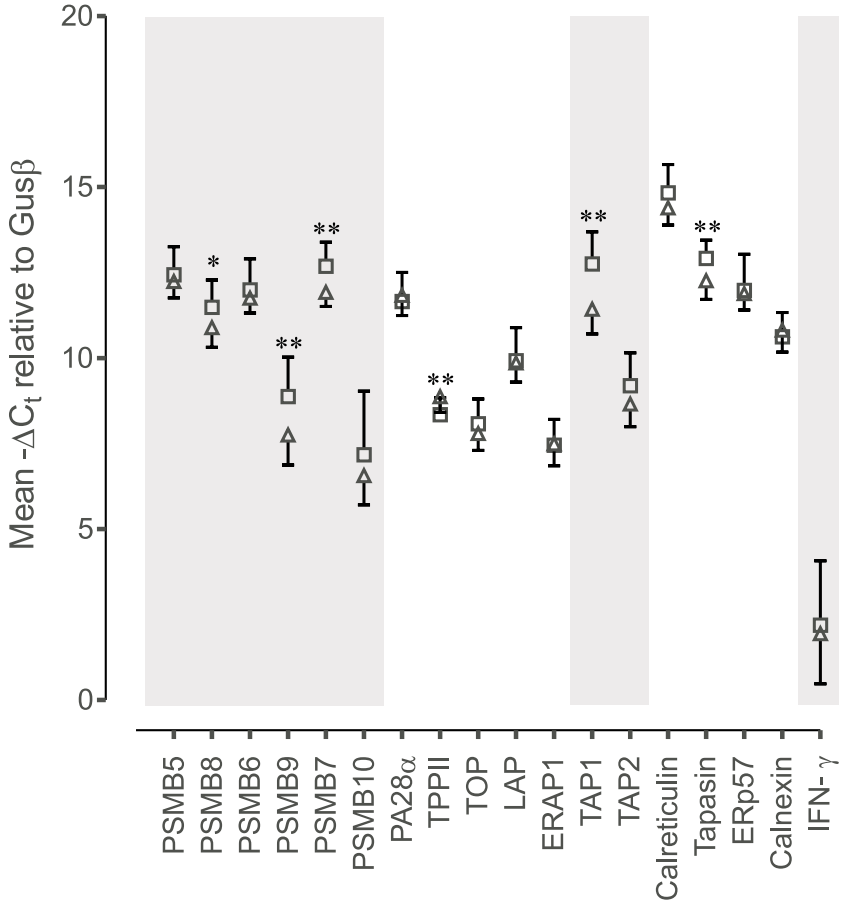

Figure 1. Overview of the mean –ΔCt values relative to Gusβ of the antigen-processing machinery genes and IFN-γ in, □ tumor tissue (n=23) and Δ control tissue (n=23). Proteasome subunits and their functional equivalents (PSMB5 through PSMB10), the TAP complex (TAP1 and TAP2) and IFN-γ are clustered by the grey area. Statistics were performed using the paired T test. The standard deviation is indicated by the lines. Significant differences between tumor and control tissue are indicated by, * ($p < 0.05$) and ** ($p < 0.01$).

The expression of the proteasome and its equivalent immunoproteasome subunits in the 23 tumor and control samples is shown, side by side, in Figure 2. PSMB5 expression was comparable between tumor and control whereas the expression of its immunoproteasome equivalent PSMB8 was significantly higher in tumor compared to control *(p = 0.044)*. PSMB6 expression did not differ between tumor and control while its immunoproteasome equivalent PSMB9 was significantly higher expressed in tumor than in control *(p = 0.004)*. Proteasome subunit PSMB7 expression in tumor was significantly higher compared to control *(p = 0.002)*. The immunoproteasome equivalent PSMB10 expression was comparable between tumor and control although in one tumor sample, the expression was much higher compared to all other tumor samples. Summarized, with the exception of PSMB10, the expression of all immunoproteasome subunits was significantly upregulated in tumor tissue. Of the proteasome subunits, only PSMB7 expression was significantly higher in tumor tissue than in control tissue (see also Figure 1). Strikingly, the gene expression of the 23 tumor samples varied more than the expression of the control samples (Figure 2).

The mean gene expression of the (immuno)proteasome activator PA28α in HNSCC was comparable with the expression in the tumor-adjacent healthy control tissue (Figure 1). Of the proteases TPPII, TOP, LAP and ERAP1, TPPII showed a significant lower mean expression *(p = 0.004)* in tumor tissue compared to control tissue (Figure 1). It is clearly shown that TPPII expression was lower in all, but three, tumors compared to control (Figure 3A). TOP expression was somewhat higher in tumor than in control tissue, whereas LAP and ERAP1 gene transcription was comparable in tumor and control tissue (Figure 1).

Of the heterodimeric TAP complex, both TAP1 and TAP2 gene expression was higher in tumor tissue compared to healthy, tumor-adjacent, control tissue (Figure 4). However, only TAP1 showed a significant difference *(p = 0.002)*. TAP2 expression was more consistent in the tumor as well as the control samples than the TAP1 expression. The mean gene expression of the ER chaperone proteins calreticulin and tapasin was higher in the tumor than in the control tissue whereas the mean expression of ERp57 and calnexin was comparable between tumor tissue and control tissue. (Figure 1). Tapasin was significantly upregulated *(p = 0.002)* in tumor tissue (Figure 3B). The mean IFN-γ expression in the tumor tissue was approximately the same as in the control tissue. However, the variation in the IFN-γ expression between the individual tumor and control samples was the largest of all genes tested (Figure 1).

For the 23 HNSCC patients, we analyzed whether there was a difference in gene expression for the APM proteins and IFN-γ between metastasizing (n=15) and non-metastasizing tumors (n=8). No statistical significant difference was found in the tumor tissue as well as in the control tissue (data not shown).

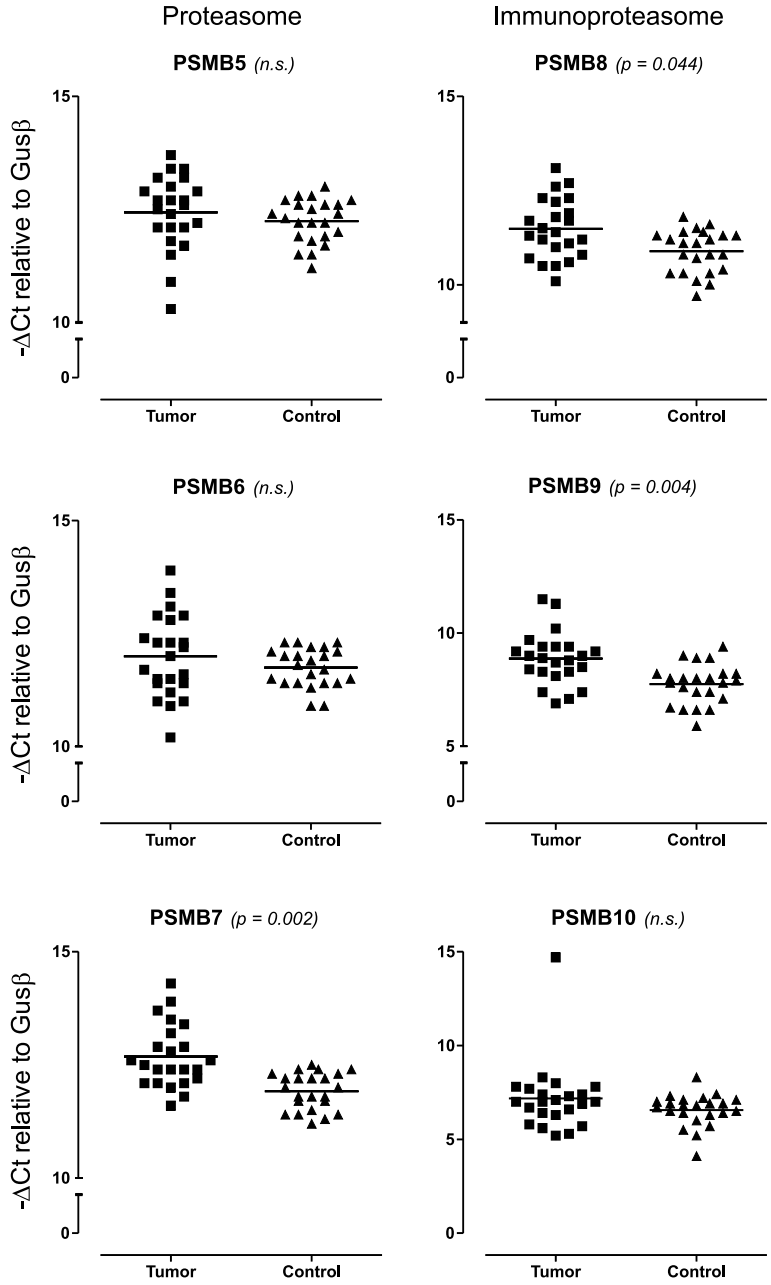

Figure 2. Gene expression of the proteasome subunits (PSMB5, PSMB6, PSMB7) and the functional equivalent immunoproteasome subunits (PSMB8, PSMB9, PSMB10) relative to Gusβ expression in 23 HNSCC and control samples. The mean expression value is indicated by a horizontal line; n.s. is not significant.

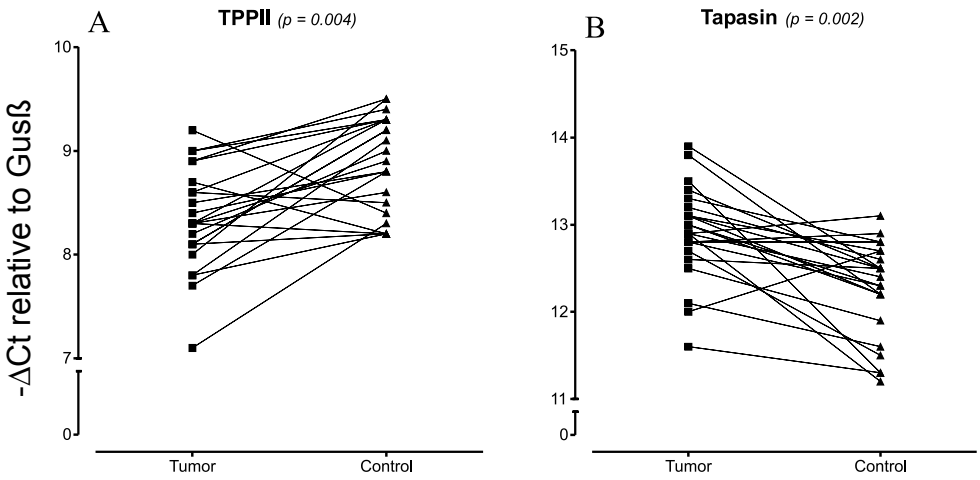

Figure 3. A) TPPII and B) tapasin –ΔCt values relative to Gusβ of the tumor (n=23) and control tissues (n=23). Pairing of the –ΔCt values of the tumors with the corresponding control tissues clearly shows the significant downregulation of TPPII and upregulation of tapasin gene expression in the tumor tissue compared to control tissue.

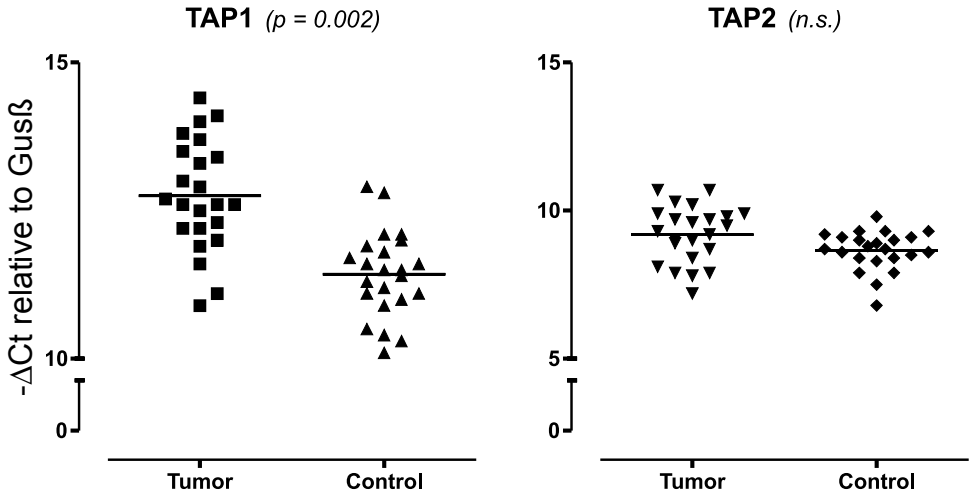

Figure 4. TAP1 and TAP2 –ΔCt values relative to Gusβ of the tumors (n=23) and control tissues (n=23). The mean expression is indicated by a horizontal line; n.s. is not significant.

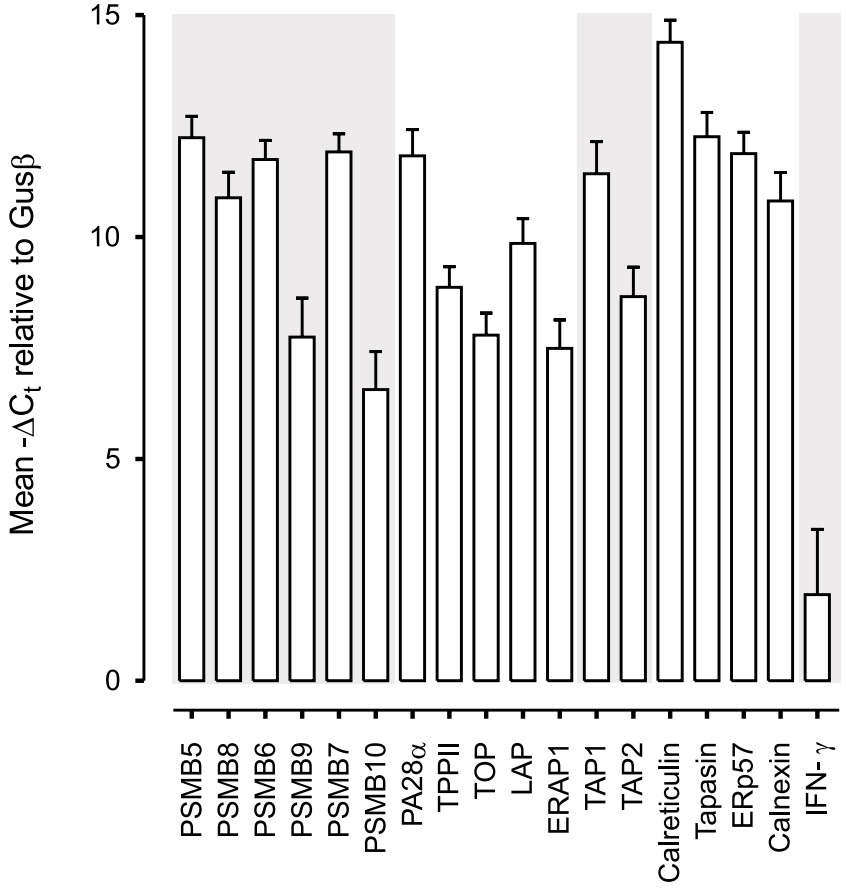

Figure 5. Expression profile of the antigen-processing machinery genes in healthy, tumor-adjacent, control tissue (n=23). Proteasome subunits and their functional equivalents (PSMB5 through PSMB10), the TAP complex (TAP1 and TAP2) and IFN-γ are clustered by the grey area. Standard deviation is indicated by the line above each bar.

The expression profile of the APM genes and IFN-γ, relative to Gusβ, in the control tissue is depicted in Figure 5. This reflects the expression level of the APM genes in healthy, tumor-adjacent, tissue. It is remarkable that all three immunoproteasome subunits (PSMB8, PSMB9, PSMB10) were expressed at a lower level than their proteasome equivalents (PSMB5, PSMB6 and PSMB7, respectively). The difference was especially large for PSMB9 and PSMB10. The proteasome activator PA28α transcription was approximately at the same level as that of the functionally related proteasome subunits. TOP and ERAP1 expression was comparable with the immunoproteasome subunit PSMB9, whereas TPPII and LAP were expressed at a higher level. Of the heterodimeric TAP complex, the transcription level of TAP2 was

considerable lower compared to TAP1. Although the mRNA expression levels of the chaperone proteins calreticulin, tapasin, ERp57 and calnexin varied, they were amongst the highest of the APM genes. In contrast, the expression level of IFN-γ was far lower compared to the APM genes.

8.4 Discussion

We quantified the RNA expression of IFN-γ and 17 genes, involved in HLA class I antigen processing, in tumor tissue and compared it with the expression in the tumor-adjacent, healthy control tissue of 23 HNSCC patients. Abnormalities in gene expression affect the antigen processing, which results in an impaired immune surveillance.

Of the proteasome subunits, only PSMB7 was significantly upregulated. This is remarkable because normally the proteasome subunits are constitutively expressed to meet the cell's need for protein turnover [40, 41]. More surprising is the fact that of all three immunoproteasome subunits, only PSMB10 is not significantly upregulated. PSMB10 is the immunoproteasome equivalent of proteasome subunit PSMB7. For the proteasome to function as an immunoproteasome, the exchange of PSMB5, PSMB6 and PSMB7 for PSMB8, PSMB9 and PSMB10 respectively, is required. How the non-significant upregulation of PSMB10 mRNA and the low mean expression level affect the formation or function of the immunoproteasome is not known.

TPPII gene expression was significantly lower in tumor than in control tissue. Since TPPII functions as a proteasome analogue, and can even substitute proteasome functions, downregulation of TPPII alters the repertoire and quantity of the peptide pool for HLA class I antigen presentation. Moreover, it is known that TPPII generates unique peptides [16, 17]. For example, the HIV-Nef (73-82) epitope is only generated by TPPII and not by the proteasome [15]. Therefore, lower expression of TPPII may represent a mechanism by which tumors can evade immune surveillance. Although the downregulation of TPPII in tumor tissue is significant, the absolute mRNA expression difference is rather small (Figure 1 and 3A). Protein-expression data is required to elucidate the significance of TPPII downregulation.

Of the heterodimeric TAP complex, both TAP1 and TAP2 were higher expressed in tumor tissue compared to control. However, only TAP1 gene expression was significantly upregulated. It is known that TAP1 and PSMB9 share a bi-directional promoter that is IFN-γ inducible [42]. This might explain the shared upregulation of TAP1 and PSMB9. Our results indicate that TAP expression is stimulated in the tumor tissue which enhances the peptide transport into the ER for HLA class I antigen presentation. This is improves immune surveillance.

The absolute mRNA expression level of TAP2 is much lower than the expression level of TAP1. The expression levels of the immunoproteasome subunits, especially

PSMB9 and PSMB10, were also lower than those of the proteasome equivalents (Figure 5). The formation and/or function of the heterodimeric TAP complex and the immunoproteasome may be hindered by these mRNA expression level differences. This depends on the translation efficiency and the protein-turnover of the individual genes. Preliminary results of TAP1, TAP2, PSMB8, PSMB9 and PSMB10 immunohistochemical staining showed normal expression at the protein level in the majority of the tumors (data not shown). Although this provides no quantitative indication of the protein expression, it demonstrates that these genes indeed are expressed at the protein level. Moreover, as we recently demonstrated, TAP1 and TAP2 polymorphisms are not involved in the development of HNSCC [43].

The significant upregulation of tapasin in tumor tissue improves the peptide loading in the ER from TAP into the HLA class I binding groove. Only when HLA class I molecules are loaded with a peptide, they are stable enough to be translocated and expressed on the cell surface. Therefore, tapasin upregulation enhances immune surveillance by stimulating HLA cell-surface expression.

Although TOP expression is not significantly higher in the tumor compared to the control tissue, it is noteworthy that overexpression of TOP destroys antigenic peptides and limits the extent of MHC class I antigen presentation [36].

The IFN-γ expression in tumor and control is comparable, but varies the most of all genes between the HNSCC patients. Although the IFN-γ gene expression in tumor and control tissue can be measured by the quantitative PCR, it is not representative for IFN-γ cytokine level in the tumor environment. IFN-γ producing immune cells surrounding the tumor are not included in the tissue sections. They are the main source of the local IFN-γ cytokine level. IFN-γ has a profound effect on APM gene expression, since the majority of the APM genes are regulated by IFN-γ.

In contrast to our study, others have shown downregulation of multiple components of the APM which was most likely caused by regulatory effects rather than structural gene defects (e.g. mutations, deletions) [31, 44]. However, these studies were performed with human carcinoma cell lines of different origin, whereas we used fresh tumor tissue sections. Tumor cell line stability could be affected by culturing. Besides, the use of β-actin as housekeeping gene is questionable. Although β-actin expression is stable in colorectal carcinoma, several studies suggested that β-actin expression is variable between different tissues, tumors and/or disease states [39, 45-48]. Cytokines, like IFN-γ, have a strong influence on APM gene expression levels. The effect of the cytokine micro-environment surrounding the tumor is neglected by cell line studies. To exclude the possibility of measuring gene expression differences between tumor and control due to differences in cytokine levels, we used tumor-adjacent healthy tissue as control. This way, the tumor tissue and control tissue shared the same cytokine micro-environment. Our results support the theory that altered APM expression in tumors is most likely caused by regulatory effects rather than

structural defects, because no major expression loss was detected in the HNSCC. Since we did not find any differences in the expression of the APM genes and IFN-γ between metastasizing and non-metastasizing HNSCC, the gene expression profile of the antigen processing machinery is not a marker for metastases.

In summary, the gene expression of proteasome subunit PSMB7, immunoproteasome subunits PSMB8 and PSMB9, TAP1 and tapasin were significantly upregulated, whereas TPPII was significantly downregulated in tumor compared to control. The upregulation of the genes in the tumor tissue suggest that the antigen processing in the tumor is improved compared to the healthy tissue. This would be beneficial for immune surveillance. However, the expression profile of the APM genes in the healthy control tissue indicated that PSMB9, PSMB10 and TAP2 mRNA expression levels were much lower compared to their functional equivalents. Whether this "normal" expression profile is sufficient for an adequate antigen processing and immune response or leads to an impaired immunoproteasome and TAP complex formation, remains to be studied. Our study shows that there were no major gene defects in the tumor tissue of HNSCC patients affecting the APM gene expression at the mRNA level. However, the downregulation of TPPII may contribute to immune surveillance evasion by the tumor cells.

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Chapter 9

General Discussion

9.1 General Discussion

Altered MHC class I antigen expression in tumors was first reported in the early 1960s [1-3]. It was soon realized that this phenomenon permits tumor cells to avoid and survive attack by the immune system. Studies of Hui *et al.* 1984 & 1989, demonstrating the loss of tumorigenicity of aggressive MHC class I negative mouse tumor cells upon transfection with MHC class I genes and the enhanced growth of mouse tumor cells with downregulated MHC class I expression, supported this notion [4, 5]. Despite the numerous studies indicating the role of MHC class I antigens restricted cytotoxic T lymphocytes (CTLs) in tumor cell eradication, interest in abnormal MHC class I antigen expression waned in the 1980s. The realization that alien histocompatibility antigens described in a number of mouse tumors and human cell lines were in fact technical artifacts, and the conflicting data regarding the clinical significance of HLA class I antigen downregulation in human tumors had a negative impact on this line of research [6, 7]. Renewed interest arose in the early 1990s with the notice of the crucial role played by MHC class I antigens in the recognition of tumor cells by CTLs [8-10]. With the identification of various TAA families and the demonstration that cytotoxic T cells have the capability of recognizing TAA and eradicating tumors in vivo, the use of T cell-based immunotherapy for the treatment of human cancer gained perspective [11-13].

Malignant melanoma has been the most extensively studied human tumor system, because of the large availability of TAA-specific T cells that can be isolated from patients with this disease. The expression of shared CTL defined melanoma-associated antigens (MAA) in a large percentage of melanoma patients enabled their use as immunogens for immunotherapy in clinical trials. A large amount of data on the immune and clinical responses that these MAA elicit, became available [14-18]. These studies showed that in the majority of patients, immunization with MAA does not yield the clinical responses predicted by murine models.

Tumor cells expressing TAA can escape TAA-specific CTL immune recognition by many factors that comprise systemic and local mechanisms. Systemic factors are immune tolerance and immune suppression. Local factors affecting the microenvironment, where tumor cell-host immune system interactions are likely to occur, can be summarized in four broad categories: i) inadequate antigen presentation by tumor cells, ii) inhibitory signals provided by the tumor microenvironment, iii) inability of TAA-specific CTLs to localize at a tumor site, and iv) inability of tumor microenvironment to sustain T cell function *in vivo*. CTL based immune escape by tumor cells can be caused by a combination of various escape mechanisms of each category. This is one of the reasons why developing an effective immunotherapy is complex [19]. Inadequate antigen presentation by tumor cells is the most elementary escape mechanism: if you can not spot the target, you can not hit it. This way, tumor cells can cloak themselves from immune surveillance by CTL. Inadequate antigen

presentation in tumor cells can be caused by: i) TAA loss or downregulation, ii) HLA class I antigen loss or downregulation, and iii) an affected functioning of the antigen presentation machinery.

9.2 TAA Loss or Downregulation

Alterations in TAA expression is one of the mechanisms by which tumor cells escape cytotoxic T cell recognition. It has been reported that deletions in the P1A gene destroy both CTL epitopes of the mastocytoma's tumor rejection antigen P815AB so that mastocytoma cells escape tumor rejection *in vivo* [20-22]. In melanoma cell lines, heterogeneity in MAA expression, independent of HLA class I antigen expression, was demonstrated [23, 24]. Although most primary melanoma lesions express MAA, metastases are quite heterogeneous [23, 25-27].

9.3 HLA Class I Loss

Many studies have been conducted to define HLA expression-loss in various tumor types and tumor cell lines [26, 28-33]. The major bottle neck in these studies has been the lack of sufficient HLA allele-specific monoclonal antibodies (mAbs). Using HLA monomorphic or locus-specific mAbs limits the details to assess and understand HLA loss in tumors. Recognizing these limitations, alternative techniques have been developed to circumvent the lack of HLA mAbs. DNA-based techniques such as loss of heterozygosity (LOH) analysis, comparative genomic hybridization (CGH) and fluorescent *in situ* hybridization (FISH) have been used to determine genomic loss of (parts of) the HLA region on chromosome 6 and β2 microglobulin on chromosome 15. Although it is well known that genomic loss is one of the mechanisms causing HLA expression-loss, it is not the only one [32, 34, 35]. Genomic presence of a gene is no guarantee for expression. Therefore, DNA-based techniques will always underestimate HLA expression-loss. Moreover, one may wonder whether these techniques can adequately assess loss of a genomic region. As we have demonstrated, LOH analysis is not conclusive for loss of heterozygosity of HLA and β2 microglobulin in the heterogeneous HNSCC (Chapters 3, 4 & 5). The complex loss patterns of the microsatellite markers studied and the fact that LOH analysis could not distinguish loss of a genomic region from gain, hindered an accurate interpretation of the LOH data. Additional FISH analysis of chromosome 6 and 15 indicated complex and heterogeneous aneuploidy in HNSCC. This illustrates the limitations of techniques such as LOH analysis and CGH. By destroying tissue morphology and investigating "pooled" tumor cell DNA, crucial information on the heterogeneous tumor is lost and results obtained, reflect an accumulation of diverse and complex tumor properties. Although tissue morphology is maintained by FISH analysis, it is difficult to determine specific HLA gene loss or allelic-loss due to the minimal requirements of the FISH-probe size and the homology between the

HLA genes. Fiber-FISH allows to detect loss of small genomic regions, but is not representative for expression. Recognizing the limitations of LOH analysis, CGH and FISH, these techniques can provide useful information. However, they are not suitable for the prediction of HLA expression in tumors.

HLA class I antigen expression is most accurately studied in surgically removed tumor specimens by immunohistochemical staining. Fresh tumor tissue is preferred above tumor cell lines because potential artifacts that may result through selection of tumor cell populations during establishment of cultured cell lines *in vitro*, are eliminated. Moreover, it allows comparison of expression levels between the tumor cells and the surrounding normal tissue and enables the detection of heterogeneous expression patterns within the tumor. Heterogeneous HLA expression within a tumor may reflect the development of escape variants. It is suggested that selective pressure by the immune system or by immunotherapy enhances the generation of malignant lesions with HLA class I defects [36]. Immune cells eliminate those tumor cell sub-populations that are amenable to the specific immune reactivity due to their HLA expression profile. Tumor cell sub-populations with a distinct HLA expression profile are left unattended and can therefore progress, expand and metastasize. Therefore, it is of crucial importance to be able to identify heterogeneous HLA expression within a tumor and its metastases.

Total HLA expression-loss can be determined by mAb W6/32 which is specific for the HLA class I/β2m complex. Immunohistochemical staining with mAb L368 is informative for β2m expression, which is crucial for stable HLA class I cell surface expression. However, total HLA expression-loss in tumors cells is not frequently found since it makes them prone for NK cell killing. HLA locus-specific mAb (e.g. HC-A2 and HC-10) are suitable to determine HLA-A and HLA-B/C locus-loss, although it should be noted that cross-reactivity with certain alleles of other loci, due to their broad specificity, can not be ruled out. The major limitation of these mAbs is that they can not adequately determine HLA expression at the allele-specific level. This is important because loss of a single HLA allele can eliminate CTL mediated recognition and eradication of tumor cells. In a murine model, it was shown that tumor cells with a selective loss of a MHC class I allele which presented an immunodominant TAA, failed to elicit a CTL immune response to other TAA presented by the expressed MHC class I alleles. Although the immunodominant TAA could not serve as a target on the tumor cells, only a CTL immune response directed to the immunodominant TAA was induced [37]. Immunodominant epitope/HLA class I combinations may have inhibitory effects on the development of CTL responses toward subdominant epitope/HLA class I combinations. Therefore, expression-loss of a dominant HLA class I antigen may provide tumor cells with a significant advantage in their progression. In our study, characterization of HLA-A and HLA-B allele-specific loss in 15 HNSCC patients demonstrated that approximately 50% of

the tumor samples had allele-specific loss. This would have remained undetected by immunohistochemical staining with HLA monomorphic and locus-specific mAbs. Moreover, at the allelic level, the HLA expression profile differed between primary lesions and metastases in more than 50% of the HNSCC patients. This emphasizes the need to study HLA expression in tumor tissue and metastases by immunohistochemical staining using HLA-allele specific mAbs. HLA typing is required to select the appropriate allele-specific mAbs for each tumor patient. Although HLA typing at the low/intermediate resolution is sufficient for the selection of mAbs, high resolution typing provides additional information on mAb specificity and strength of TAA binding. This information is especially useful for improvement of immunotherapy. By determining the HLA alleles that are expressed in tumor cells, one can predict those TAA that can be presented, which allows the development of TAA-specific T cell based immunotherapy.

9.4 HLA Down- or Upregulation.

Although HLA downregulation is associated with tumor progression, this does not hold true for all tumors. In HNSCC, cutaneous melanoma, breast-, prostate- and bladder carcinoma, HLA class I antigen defects are associated with poor prognosis, whereas in some tumors it is associated with favorable prognosis [38]. In cutaneous melanoma where CTL are believed to control the metastatic tumor spread via the lymphatics, HLA class I antigen downregulation is associated with a poor prognosis [39]. In contrast, HLA class I antigen downregulation is associated with a favorable clinical outcome in uveal melanoma, where NK cells, which tend to kill tumor cells with a low HLA class I antigen expression, have been suggested to limit metastases via the blood [40, 41]. This illustrates the balance in HLA expression tumors have to find to evade CTL and NK cell responses. Moreover, it shows that both CTL and NK cells are capable of tumor cell eradication. One may consider whether immunotherapy should be directed to restore HLA expression for a CTL mediated response, or to induce HLA expression downregulation that may induce a NK cell mediated response.

9.5 HLA Class II Expression

It is currently believed that tumor cell recognition and eradication is mainly facilitated through HLA class I molecules. However, HLA class II expression in tumor cells is gaining more and more interest. In a panel of over 100 HNSCC, we demonstrated HLA class II expression by immunohistochemical staining in approximately 50% of the tumors (Chapter 2). Squamous cells normally are devoid of HLA class II molecules. This indicates potential involvement of HLA class II molecules in tumor progression. In melanoma, HLA class II antigen associated MAA have been identified and there is evidence that malignant cells can present endogenous antigens

in association with HLA class II molecules [42-46]. Activation and proliferation of naive cytotoxic T cells depends on cytokines that are produced by Th-1 helper cells which in turn are activated through HLA class II expression [47]. There is evidence that CD4+ T cells not only play a role in anti-tumor immune responses by modulating T and B cell responses, but also by direct eradication of neoplastic cells [48, 49]. CD4+ T cells with regulatory properties (Treg cells) are divided into two subtypes, natural and adaptive, according to their ontogeny and mode of action [50-52]. Adaptive Treg cells demonstrated cytotoxicity against allogeneic tumor cell lines in a perforin-dependent, but MHC/TCR-independent, manner [49, 53]. Moreover, it was shown that both subtypes of Treg cells exhibit perforin-dependent cytotoxicity against a variety of autologous target cells, including CD4+ and CD8+ T cells and dendritic cells. Activated CD4+ and CD8+ T cells were preferentially killed over unactivated T cells [49]. On the other hand, it has been demonstrated that HLA-DR signaling activates the MAPK/Erk pathway in A375 melanoma cells, which has a functional role in the resistance of these cells to Fas-mediated apoptosis [54]. This underlines the potential importance that HLA-DR signaling might have in melanoma immune escape and tumor progression. However, it has also been reported that downregulation of HLA-DR in gastrointestinal cancer cells and in a subset of haematopoietic tumors enables tumors to escape immune surveillance [55, 56]. This downregulation of HLA-DR was caused by methylation of the MHC class II coactivator, CIITA-PIV. Hypermethylated DNA generally is transcriptional inactive, whereas actively transcribed genes contain low levels of DNA methylation at their promoter region. It has been shown that cancer cells show altered patterns of methylation which may contribute to aberrant HLA expression and tumor development [57].

It is clear that HLA class II expression may exert many effects through varied mechanisms that may stimulate tumor eradication as well as tumor progression. It is not known which mechanisms are functional and effective, nor whether HLA class II expression in tumor cells plays a significant role in the modulation of host immune recognition of tumor cells.

9.6 Antigen Processing and Presentation

Apart from HLA molecules, β2m and peptides, adequate processing is required to modify and assemble the HLA class I/β2m/peptide complex for stable cell surface expression. Although a number of molecules involved in the antigen processing have been identified, earlier studies have been primarily restricted to TAP1, TAP2, PSMB8 and PSMB9 [58-65, 66, 67]. Defects in the comprehensive and complex antigen-processing machinery affects the presentation of TAA by HLA class I molecules. It has been shown that altered expression of PSMB and TAP subunits causes defective processing and presentation of antigenic peptides to specific CTL

[68-71]. We investigated mRNA expression of the majority of genes involved in HLA class I antigen processing in 23 HNSCC. Our results indicated that the gene expression of the APM components in the tumors is comparable with, or higher than the gene expression in tumor-adjacent healthy tissue. The gene expression of PSMB7, PSMB8, PSMB9, TAP1 and tapasin was significantly upregulated, whereas TPPII gene expression was significantly downregulated in tumor compared to control tissue. TPPII downregulation may alter the composition of the peptide pool available for HLA class I binding [72-74]. In contrast to our study, others have found downregulation of TAP and PSMB genes in tumor cell lines [67, 71, 75-77]. One may question how representative these results are for the *in vivo* situation. In the majority of the tumor cell lines, the expression defects were reversible by stimulation of the tumor cell line cultures with IFN-γ. The expression downregulation may therefore reflect differences in the cytokine micro-environment between the *in vivo* and *in vitro* situation. As no gene expression defects were detected in our study, expression regulation factors, e.g. cytokines, are likely to be responsible for altered APM gene expression in HNSCC. However, mutations in APM genes also affect antigen processing and presentation. The first evidence for a naturally occurring protein structural defect resulting in defective peptide transport in a human solid tumor was demonstrated by Chen *et al.* 1996 [78]. However, more and more studies report mutations and gene defects in TAP1 and TAP2 [79-82].

Although the expression and function of many APM components is critical, TAP is considered to be one of the most critical and has been frequently studied [63]. Seliger *et al.* 1997, reviewed TAP expression in tumors as "TAP off-tumors on" [83]. Although it has been demonstrated that TAP expression is not required for HLA class I cell surface expression, it is essential for adequate antigen presentation. Without TAP, cytoplasmic located TAA can not enter the ER and can therefore not be presented on the cell surface. Signal leader peptides enter the ER in a TAP independent way and bind to HLA class I molecules for cell surface expression. Thus, HLA class I expression is maintained, but the autologous signal peptides do not have the capability to elicit a CTL immune response. By TAP expression-loss, tumors are not susceptible to NK cell mediated lysis due to maintained HLA class I expression and are resistant to CTL mediated eradication due to the lack of immunogenic TAA presentation. In this way, tumor cells evade both arms of the cellular immune surveillance.

9.7 Implications for Immunotherapy.

There are many ways by which tumor cells evade immune surveillance ranging from systemic to local mechanisms. Tumor evasion is rarely caused by one single defect, but more likely by a combination of various escape mechanisms, which may even differ between patients. Moreover, the multiple distinct escape mechanisms

are functionally related. This makes it hard to comprehend how tumors escape immune surveillance and are able to progress in immunocompetent patients. Designing effective immunotherapy is a daunting task. Clinical phase I trials with immunotherapy in melanoma have been conducted, but the outcome was not as promising as suspected. The major benefit of immunotherapy is the capability of the immune system to recognize and eradicate all neoplastic cells by their distinct presentation of TAA through HLA class I molecules, allowing total tumor eradication with a minimum of side effects. The problem with current local treatment, such as surgery and radiation, is the difficulty to target all tumor cells in a patient. This is important since any tumor cells left may progress and develop into secondary tumors or metastases. Chemotherapy works systemic but is not specific for neoplastic cells which results in severe side effects. Since immune surveillance works systemic and can specifically target neoplastic cells, immunotherapy would be an ideal treatment for cancer patients. As mentioned before, the efficacy of immunotherapy largely depends on the presentation of TAA by HLA class I molecules. The fact that HLA expression is frequently abrogated in a wide variety of tumors hinders an adequate functioning of T cell-based immunotherapy. Application of T cell-based immunotherapy in tumors with a heterogeneous HLA expression profile may even have adverse effects. Tumor cells expressing the restrictive TAA by a specific HLA allele are eradicated by immunotherapy, but sub-populations of the heterogeneous tumor with another HLA expression profile can not be recognized and eradicated. These tumor cells remain unattended by the immune response and can progress freely. In fact, the pressure from either the immune system or immunotherapy drives the tumor to expand its sub-populations that are not susceptible for the immune pressure. In this way, tumor escape variants progress that become more and more difficult to beat. One may compare this process with the ongoing battle between antibiotics and resistant pathogens. The superfluous, non-specific use of antibiotics drives pathogens to evolve resistant strains by "mutational-evolution". Random mutations in the genome of tumor cells, due to genetic instability, provides tumor cells with adaptive properties. The heterogeneous HLA class I expression, which was frequently demonstrated in our studies, may represent the result of this escape mechanism. Therefore, it is important to characterize HLA class I expression at the allelic level by immunohistochemical staining in fresh tumor samples before immunotherapy is used. Immunotherapy is only effective when it can hit all tumor cells. This requires uniform expression of TAA by HLA class I molecules throughout the tumor and metastases.

Although each tumor type and even each tumor patient may have its own combination of defects leading to tumor progression and immune evasion, the basic requirement for T cell-based immunotherapy is appropriate TAA presentation by HLA antigens on tumor cells. Due to the lack of sufficient allele-specific mAbs, HLA

expression-loss has not yet been adequately assessed in the majority of tumors. Our studies shed the first ray of light on allele-specific HLA expression-loss in tumor tissue and metastases of HNSCC patients. To elucidate the role of HLA expression in tumor progression and immune evasion, this line of research needs to be continued not only in HNSCC, but also in any other tumor type. Providing the immune system's army with "smart bombs" does not win the war when the target can not be spotted.

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Chapter 10

Summary

10.1 Summary

Cytotoxic T cells target tumor cells that present tumor associated antigens (TAA) by human leukocyte antigen (HLA) molecules on the cell surface. The generation of TAA is a crucial process since the TAA bound by HLA class I molecules on the cell surface elicit the cytotoxic T cell response. HLA class I antigen processing is a comprehensive and complex machinery in which defects can result in sub-optimal immune surveillance. In tumors, loss of HLA expression is frequently observed. An aberrant expression of HLA on the cell surface affects the efficacy of immune surveillance by cytotoxic T cells and innate natural killer (NK) cells. This way, tumor cells hide themselves from immune surveillance and escape eradication by immune cells. Moreover, loss of HLA expression limits the applicability and efficacy of immunotherapy.

The aim of our studies was to characterize HLA class I expression in head and neck squamous cell carcinoma (HNSCC) and study underlying mechanisms of HLA loss. Secondly, we investigated the role of the HLA class I antigen-processing machinery in the development of HNSCC.

HLA class I and class II expression of 102 HNSCC was characterized by immunohistochemical staining using monoclonal antibodies HC-A2, HC-10, L368 and LGII-612.14 that recognize monomorphic determinants of HLA-A, HLA-B/C, β2 microglobulin and HLA class II, respectively. The expression of β2m is essential for stable HLA class I cell surface expression. In 57% of the HNSCC, β2m expression was affected, while in approximately 20%, immunohistochemical staining of β2m was completely negative. In HNSCC with negative β2m expression, we performed sequencing based mutation analysis for the β2m exons and surrounding intron regions, but no mutations were found. Immunohistochemical analysis with mAb HC-A2 demonstrated that in 95% of the HNSCC HLA-A expression was affected of which 26% had HLA-A locus-loss. HC-10 staining showed that HLA-B/C expression was affected in approximately 75% of which approximately 15% had HLA-B/C locus-loss. Heterogeneous HLA expression was frequently found in the HNSCC lesions. HLA-A expression seemed to be affected more than HLA-B and HLA-C combined. This was not supported by our study with allele-specific mAbs. Individual loss percentages of HLA-B or HLA-C could be higher. HLA-B and HLA-C locus specific antibodies are required to elucidate this question. Due to cross-reactivity of HC-A2 and HC-10, the use of these antibodies to determine HLA class I expression-loss is of limited value. Strikingly, 45% of the HNSCC showed some degree of HLA class II staining. Whether and how HLA class II expression is involved in the development of HNSCC remains to be studied. Clearly, HLA class I expression is severely diminished in HNSCC, partially by loss of β2m expression.

Microsatellite markers are frequently used to study loss of heterozygosity (LOH) of β2m. We re-evaluated the microsatellite markers suitable for β2m LOH analysis in sight of the new information on the human genome. Comparing the β2m LOH data with the β2m immunohistochemical data raised doubts about the interpretation of LOH analysis in HNSCC. LOH analysis with microsatellite markers could not adequately define β2m LOH mainly because genomic loss and gain could not be distinguished. Fluorescent *in situ* hybridization (FISH) analysis was used to elucidate whether LOH may represent loss as well as gain.

FISH analysis of chromosome 15, and chromosome 1 as control, was performed in 11 HNSCC to determine whether β2m LOH analysis is representative for genomic loss. For five HNSCC lesions with LOH and β2m expression, chromosome 15 FISH indicated gain rather than loss. In the majority of the 11 HNSCC lesions, FISH showed chromosome 1 and 15 heterogeneity throughout the tumor. Moreover, FISH indicated complex chromosome 1 and 15 aneuploidy. Our results showed that microsatellite LOH analysis did not represent the β2m gene copy number and can not predict expression. Conclusions on genomic alterations in tumors can not be based on LOH data only, but depend on the results of immunohistochemical staining, FISH or comparative genomic hybridization (CGH) analysis.

Chromosome 6 FISH analysis was performed in nine HNSCC to determine whether LOH analysis was representative for genomic HLA loss. FISH analysis showed that only two tumors with LOH and one without LOH indeed had loss and a normal chromosome 6 distribution, respectively. For the remaining six tumors, LOH analysis did not reflect the genome HLA copy number. We demonstrated that LOH analysis can not distinguish loss from gain because the HLA region was not homogeneously affected within a tumor. Tumor heterogeneity and complex aneuploidy in tumors hinder a straightforward and accurate interpretation of microsatellite marker analysis.

From these studies, it can be concluded that HLA expression is frequently and severely affected in HNSCC. Genomic loss of β2m or HLA partially accounts for the aberrant HLA cell surface expression. Although microsatellite marker LOH analysis is a quick and easy technique to study LOH, it is not applicable in HNSCC due to the heterogeneous and complex aneuploidy. For immune therapy strategies in cancer patients, knowledge of the HLA expression on tumor cells is essential, to which LOH analysis has a limited contribution.

To determine HLA cell surface expression, HLA specific antibodies, and in particular allele-specific mAbs, are essential. We determined HLA-A and HLA-B allele-specific expression in HNSCC lesions and metastases of 15 patients with HNSCC by immunohistochemical staining. Allele-specific HLA loss was demonstrated in approximately 50% of the tumors which would have remained undetected with locus-specific or monomorphic HLA antibodies. The HLA-B8 allele

expression in five HNSCC patients, which have the autoimmune disease associated HLA-B8/DR3 haplotype, was affected. Moreover, over 50% of the tumor patients had a distinct HLA expression-loss profile in the primary lesion compared to the metastasis. HLA typing was crucial to select the allele-specific mAbs and to define the specificity of the allele-specific mAbs. HLA allele-specific mAbs and HLA typing are required to gain more insight in HLA expression in tumors.

Since only a minority of HNSCC showed total expression-loss of the classical HLA class I genes, the majority of tumors still may have functional antigen presentation. Therefore, we investigated whether the antigen-processing machinery plays a role in the loss or downregulation of TAA presentation. Polymorphisms in the heterodimeric TAP complex may contribute to peptide-selective transport. This would influence the availability of TAA in the endoplasmic reticulum (ER) for presentation by HLA class I. We developed a high-throughput SNaPshot™ assay for TAP1 and TAP2 allele typing and determined the TAP1 and TAP2 allele frequencies in 94 HNSCC patients and 79 Dutch control individuals. Strikingly, all control subjects and HNSCC patients contained a TAP1*0101 allele, with the exception of one tumor patient. The most frequent alleles were TAP1*0101 (88.2%) and TAP2*0101 (81.2%). No significant difference was found between control subjects and HNSCC patients or between metastasizing and non-metastasizing tumors. In this study, we defined the TAP1 and TAP2 allele frequencies in the Dutch population and identified the new TAP1*0501 allele.

The HLA class I antigen processing machinery (APM) consists of multiple components involved in peptide generation by protein degradation, peptide transport into the ER and peptide assembly with HLA class I molecules. Distinct components of the APM have been studied in a variety of tumors. Downregulation of APM genes rather than structural defects accounted for the expression-loss in the majority of carcinoma or tumor cell lines. We studied the gene expression at the RNA level for all currently known APM genes and the immune regulatory cytokine IFN-γ in tumor tissue of 23 HNSCC patients, compared to the expression in healthy tissue of the patients. A significant upregulation of PSMB7, PSMB8, PSMB9, TAP1, tapasin and a significant downregulation of TPPII gene expression in tumors was identified. Moreover, in tumor as well as in control, the expression levels of PSMB9, PSMB10 and TAP2 were much lower compared to their functional equivalents PSMB6, PSMB7 and TAP1, respectively. Overall, the gene expression profile of the other APM genes in the tumor tissue of the 23 HNSCC patients was approximately the same or higher compared to the control tissue. No downregulation, except for TPPII, was found. Although protein expression and functionality of the APM remains to studied, our data supports the theory that downregulation or expression-loss of APM genes is likely to be caused by regulating factors rather than gene defects.

From this study it can be concluded that immunotherapy, targeting HNSCC via HLA class I presented TAA, is still possible. However, the aberrant HLA expression reduces the efficacy of the therapy. Whether HLA class I expression can be upregulated, depends on the defects that cause the aberrant HLA expression. Moreover, the variety of HLA expression-loss in each HNSCC patient requires a tailored treatment. For this, it is important to determine the precise HLA class I cell surface expression with allele-specific mAbs. The use of "patient non-specific immunotherapies" may drive heterogeneous HNSCC to expand "escape variants".

Chapter 11

Appendix

Nederlandse Samenvatting

Hoofd- en halstumoren ontstaan uit de epitheelcellen die het slijmvlies in de keel- en mondholte bekleden. Het is wereldwijd de vijfde meest voorkomende kankersoort die jaarlijks 780.000 nieuwe gevallen telt. Alcohol- en tabaksgebruik zijn de voornaamste risicofactoren waardoor deze tumoren kunnen ontstaan. Ofschoon de incidentie voor mannen hoger ligt, neemt het aantal vrouwen dat hoofd- en halskanker krijgt toe. De oorzaak hiervan ligt in het toenemende alcohol- en tabaksgebruik onder vrouwen.

De behandeling van hoofd- en halskankerpatiënten is de afgelopen decennia niet noemenswaardig verbeterd. Ondanks verbeteringen in diagnose- en behandelingsmethoden, zoals chirurgie, bestraling en chemotherapie, is de vijfjaars overlevingskans de afgelopen 30 jaar lager dan 50% gebleven. Alleen bij een vroege ontdekking van de tumor kan de patiënt goed behandeld worden. Echter, bij het merendeel van de patiënten wordt de tumor pas in een laat stadium gediagnostiseerd.

De ontwikkeling van immunotherapie heeft de laatste jaren steeds meer aandacht gekregen als mogelijkheid om de overleving van patiënten met hoofd- en halskanker te verbeteren. Met immunotherapie wordt het immuunsysteem van de patiënt gestimuleerd en/of geactiveerd om de tumorcellen te vernietigen. Er wordt gebruik gemaakt van de capaciteit en effectiviteit van het menselijke immuunsysteem om, net als bij bijvoorbeeld een griepinfectie, de zieke cellen op te ruimen. Het voordeel boven de huidige behandelingsmethoden is dat met immunotherapie alle tumorcellen specifiek kunnen worden vernietigd. Hiermee wordt voorkomen dat enkele tumorcellen de behandeling overleven en zich weer verder ontwikkelen tot nieuwe tumoren. De toepassing van immunotherapie is nodig, omdat tumoren de functionaliteit van het immuunsysteem kunnen belemmeren. Daarnaast kunnen tumorcellen onzichtbaar worden voor de cellen van het immuunsysteem. Immuuncellen, met name cytotoxische T-cellen, herkennen tumorcellen via humane leukocyte antigenen (HLA) op het tumorceloppervlak. De HLA klasse I moleculen presenteren tumor geassocieerde antigenen (TAA) die niet voorkomen in normale gezonde cellen. Hierdoor kunnen de cytotoxische T-cellen heel specifiek de tumorcellen opruimen. Wanneer tumorcellen geen HLA klasse I moleculen meer op hun celoppervlak hebben, kunnen ze niet worden herkend en dus niet meer worden opgeruimd door de cytotoxische T-cellen. Daarentegen vernietigen de “natural killer” (NK) cellen van het immuunsysteem alle cellen die geen of te weinig HLA klasse I moleculen op het celoppervlak hebben. Sommige tumorcellen vinden een balans tussen HLA klasse I expressie en HLA klasse I verlies, waardoor zowel de cytotoxische T-cellen als de NK-cellen ze niet kunnen vernietigen. Hierdoor ontsnappen tumoren niet alleen aan het immuunsysteem, maar zijn ze ook minder vatbaar voor immunotherapie. Voor

het ontwikkelen van effectieve immunotherapieën is het van belang de mogelijkheid van presentatie van TAA door HLA klasse I moleculen op het tumorceloppervlak in kaart te brengen.

Het doel van onze studies, beschreven in dit proefschrift, was om HLA klasse I expressie, en de onderliggende mechanismen voor verlies van HLA-expressie, in hoofd- en halstumoren te bestuderen. Daarnaast hebben we de rol van het TAA productiemechanisme in de ontwikkeling van hoofd- en halstumoren onderzocht.

HLA-expressie in 102 hoofd- en halstumoren werd in kaart gebracht door middel van immuunhistochemische kleuringen met de monoclonale antilichamen HC-A2, HC-10, L368 en LGII-612.14 die respectievelijk HLA-A, HLA-B/C, β2 microglobuline (β2m) en HLA klasse II antigenen aantonen. De expressie van β2m is essentieel voor een stabiele HLA klasse I expressie op het celoppervlak. β2m-expressie was in 57% van de hoofd- en halstumoren aangetast, terwijl in ongeveer 20% de expressie totaal verloren was. Om te kijken of dit expressieverlies werd veroorzaakt door mutaties in het β2m-gen hebben we de tumoren zonder β2m-expressie onderzocht met behulp van DNA-sequentie analyse. Er werden geen mutaties gevonden. HLA-A expressie was aangetast in 95% van de tumoren waarvan 26% totaal verlies van HLA-A expressie had. Voor HLA-B/C expressie was dit respectievelijk 75% en 15%. Heterogene HLA-expressie werd vaak geobserveerd in hoofd- en hals tumoren. Deze data tonen aan dat HLA-A expressie frequenter is aangedaan dan HLA-B en HLA-C expressie gecombineerd. Onze studie met HLA allelspecifieke antilichamen toonde echter aan dat HLA-B expressie frequenter is aangedaan dan HLA-A expressie. Deze tegenstrijdigheid kan worden verklaard door het gegeven dat antilichaam HC-10 zowel HLA-B als HLA-C aantoont. Individuele verliespercentages voor HLA-B en HLA-C kunnen hoger liggen. Om dit uit te zoeken zijn HLA-B en HLA-C locusspecifieke antilichamen noodzakelijk. Het gebruik van de antilichamen HC-A2 en HC-10 zijn van beperkte waarde om HLA klasse I expressie in kaart te brengen, omdat ze kruisreactiviteit vertonen met andere HLA-antigenen. Opvallend was dat 45% van de hoofd- en halstumoren HLA klasse II expressie vertoonden aangezien ze dit normaal niet hebben. Hoe en of de expressie van HLA klasse II van invloed is op de tumorontwikkeling blijft de vraag.

Microsatellietmarkers worden frequent gebruikt om verlies van heterozygotie (LOH) van β2m te bepalen. Wij hebben de microsatellietmarkers die geschikt zijn voor β2m LOH-analyse geëvalueerd, omdat nieuwe informatie over het humane genoom beschikbaar kwam. Vergelijking van de β2m LOH data met de data verkregen uit de β2m immunohistochemische kleuringen wekte twijfel op over de interpretatie van de LOH-analyse. LOH-analyse met behulp van microsatellietmarkers kan geen onderscheid maken tussen verlies en amplificatie van een genomisch gebied. Daarom werd fluorescente *in situ* hybridisatie (FISH) toegepast om te bepalen of β2m LOH-analyse verlies of amplificatie aantoonde.

FISH-analyse voor chromosoom 15, waar β2m gelokaliseerd is, en chromosoom 1, als controle, werd verricht op 11 hoofd- en halstumoren om te bepalen of β2m LOH-analyse representatief is voor genomisch verlies van β2m. Voor vijf hoofd- en halstumoren met β2m-LOH en β2m-expressie op het celoppervlak, toonde de FISH-analyse amplificatie in plaats van verlies van chromosoom 15 aan. In de meerderheid van de 11 tumoren gaf de FISH-analyse heterogene en complexe aneuploidie aan voor zowel chromosoom 15 als chromosoom 1. Hieruit kan geconcludeerd worden dat LOH-analyse niet representatief is voor het aantal β2m gen kopieën. Alleen in combinatie met andere technieken, zoals immunohistochemische kleuringen, FISH en "comparative genomic hybridization" (CGH) kunnen er conclusies getrokken worden.

Chromosoom 6 FISH-analyse werd verricht op negen hoofd- en halstumoren om te bepalen of LOH-analyse representatief is voor genomisch verlies van het HLA gebied. Voor zes van de negen tumoren kwam de LOH data niet overeen met de FISH data. Door middel van de FISH-analyse konden we aantonen dat het HLA gebied niet homogeen aangetast is binnen een tumor waardoor LOH-analyse geen onderscheid kan maken tussen verlies en amplificatie van het HLA gebied. Geconcludeerd kan worden dat heterogeniteit en complexe aneuploidie in een tumor een recht toe recht aan en accurate interpretatie van microsatellietmarker LOH-analyse belemmert.

Samenvattend kunnen we stellen dat HLA-expressie vaak en ernstig is aangetast in hoofd- en halstumoren. Verlies van β2m expressie is hier deels de oorzaak van. Ofschoon microsatellietmarker LOH-analyse een snelle en eenvoudige techniek is, is deze ongeschikt om toe te passen in heterogene tumoren met complexe aneuploidie zoals hoofd- en halstumoren. Voor immunotherapiestrategieën in kankerpatiënten is kennis van HLA-expressie op het celoppervlak essentieel. Hiertoe levert LOH-analyse een beperkte bijdrage.

Om HLA-expressie op het celoppervlak adequaat te kunnen bestuderen, zijn HLA allelspecifieke antilichamen noodzakelijk. Wij hebben HLA-A en HLA-B allelspecifieke expressie bepaald met behulp van humane monoclonale HLA allelspecifieke antilichamen in de primaire tumor en metastase van 15 hoofd- en halskankerpatiënten. HLA allelspecifiek expressieverlies werd aangetoond in 50% van de tumoren. Dit verlies zou onopgemerkt blijven wanneer, zoals gebruikelijk, alleen HLA locusspecifieke of monomorfe HLA-antilichamen waren gebruikt. In vijf patiënten met het auto-immuunziekte geassocieerde haplotype HLA-B8/DR3, was de HLA-B8 expressie aangedaan. Daarnaast verschilde het HLA allelspecifieke expressieprofiel tussen de primaire tumor en de bijbehorende metastase in meer dan 50% van de hoofd- en halstumorpatiënten. HLA-typering was cruciaal om de allelspecifieke antilichamen te selecteren en hun specificiteit nauwkeuriger te

definiëren. Geconcludeerd kan worden dat HLA allelspecifieke antilichamen en HLA typering beide noodzakelijk zijn om meer inzicht te krijgen in HLA-expressie in tumoren.

Aangezien maar een klein percentage van de tumoren totaal HLA-verlies hebben, heeft de meerderheid van de hoofd- en halstumoren nog de mogelijkheid om TAA te presenteren. Dit vereist wel een functioneel antigen producerend mechanisme (APM). Het "transporter associated with antigen processing" (TAP)-complex is hier een cruciaal onderdeel van. Polymorfismen in de TAP1- en TAP2-genen, die coderen voor het heterodimere TAP-complex, zouden de beschikbaarheid van TAA in het endoplasmatisch reticulum (ER) voor presentatie door de HLA klasse I moleculen kunnen beïnvloeden. Wij ontwikkelden een SNaPshot™ techniek om snel en eenvoudig de TAP1- en TAP2-allelen te typeren van 94 hoofd- en halskankerpatiënten en 76 Nederlandse bloeddonoren als controle. Alle individuen hadden een TAP1*0101 allel met uitzondering van één kankerpatiënt. De meest frequente allelen waren TAP1*0101 (88,2%) en TAP2*0101 (81,2%). We vonden geen significant verschil in TAP1- en TAP2-allelfrequenties tussen de kankerpatiënten en de controles. In deze studie hebben we de TAP1- en TAP2-allelfrequenties in de Nederlandse populatie gedefinieerd en een nieuwe (TAP1*0501) allel geïdentificeerd.

Het HLA klasse I APM bestaat uit meerdere componenten met een verscheidenheid aan functies. Een defect in één van deze onderdelen heeft een groot effect op de productie van TAA die gepresenteerd kunnen worden door de HLA klasse I moleculen. Wij bestudeerden de gen-expressie op RNA-niveau van genen die coderen voor de APM-componenten en het immuunregulatoire cytokine interferon-γ (IFN-γ) in tumorweefsel van 23 hoofd- en halskankerpatiënten en vergeleken dit met de expressie in het gezonde weefsel van de patiënten. Er werd een significante toename van "proteasome subunit β" (PSMB)7, PSMB8, PSMB9, TAP1, tapasin en een significante afname van TPPII-expressie in tumorweefsel ten opzichte van gezond weefsel gevonden. De absolute expressieniveaus van PSMB9, PSMB10 en TAP2 waren veel lager in zowel het tumorweefsel als het gezonde weefsel dan hun functionele equivalenten PSMB6, PSMB7 en TAP1. In het algemeen is het gen-expressiepatroon van de APM-genen en IFN-γ vergelijkbaar of hoger in tumorweefsel vergeleken met gezond weefsel. Behalve voor TPPII, is er geen afname in gen-expressie gevonden. Ofschoon gen-expressie niets zegt over de functionaliteit van het APM, onderschrijven deze data de theorie dat expressie-afname of expressieverlies van APM-genen meest waarschijnlijk wordt veroorzaakt door regulerende factoren in plaats van gendefecten.

Uit onze studies blijkt dat immunotherapie via HLA gepresenteerde tumorantigenen nog altijd mogelijk is. Echter, de aangetaste HLA-expressie beïnvloedt wel de effectiviteit van de therapie. Of de HLA-expressie kan worden hersteld, hangt af van de oorzaak van het HLA-expressiedefect. De verscheidenheid

aan HLA-expressieverlies in elke hoofd- en halskankerpatiënt vereist een op het individu gerichte behandeling. Hiervoor is het van belang dat de HLA-expressie op het celoppervlak van tumoren en metastasen exact wordt bepaald door middel van HLA allelspecifieke antilichamen. Het toepassen van "niet-patiënt specifieke" immunotherapie op heterogene hoofd- en halstumoren kan leiden tot het ontwikkelen van ontsnappingsvarianten.

List of Publications

1. Koene GJPA, van der Ven KJW, Verdaasdonk MAM, Slootweg PJ, de Weger RA, Tilanus MGJ: A Variety of HLA Phenotypes in Head and Neck Squamous Cell Carcinoma Patients Identified with the Workshop Antibodies as defined in the HLA and Cancer Component. *In Hansen J, Dupont B (eds): HLA 2004: Immunobiology of the Human MHC. Proceedings of the 13th International Histocompatibility Workshop and Congress.,* Vol I & II. Seattle, WA, IHWG Press, 2005.

2. Koene GJPA, Coenen S, Verdaasdonk MAM, de Weger RA, Slootweg PJ, Tilanus MGJ: The value of loss of heterozygosity of short tandem repeat markers to define expression variants of the β2-microglobulin gene. *In Hansen J, Dupont B (eds): HLA 2004: Immunobiology of the Human MHC. Proceedings of the 13th International Histocompatibility Workshop and Congress,* Vol I & II. Seattle, WA, IHWG Press, 2005.

3. Koene GJPA, Arts-Hilkes YHA, van der Ven KJW, Rozemuller EH, Slootweg PJ, de Weger RA, Tilanus MGJ: High level of chromosome 15 aneuploidy in Head and Neck Squamous Cell Carcinoma lesions identified by FISH analysis: limited value of ß2 Microglobulin LOH analysis. *Tissue Antigens* 64:452, 2004.

4. Koene GJPA, Arts-Hilkes YHA, van Dijk AJG, van der Ven KJW, Slootweg PJ, de Weger RA, Tilanus MGJ: High level of aneuploidy of chromosome 6 by FISH analysis of head and neck squamous cell carcinoma: Limited applicability of LOH analysis to define HLA loss. *Human Immunology* 65:1455, 2004.

5. Koene GJPA, Mulder A, van der Ven KJW, Eijsink C, Franke M, Slootweg P, Claas FH, Tilanus MGJ: HLA Class I Allele-specific Expression-loss in Head and Neck Squamous Cell Carcinoma and Corresponding Lymph Node Metastases. *Submitted.*

6. Koene GJPA, v/d Weide P, Arts-Hilkes YHA, Bastiaans E, Rozemuller EH, Slootweg PJ, Tilanus MGJ: TAP1 and TAP2 Allele Frequencies in a SNaPshot™: no evidence for allelic prevalence in HNSCC Patients compared to Dutch Controls. *Human Immunology* 66:77, 2005.

7. Koene GJPA, van Dijk JJG, van Grinsven KWA, Slootweg PJ, Tilanus MGJ: Gene Expression Profile of HLA Class I Antigen Processing Genes in Head and Neck Squamous Cell Carcinoma. *Submitted.*

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"Elk afscheid betekent de geboorte van een herinnering."
Salvador Dalí

Curriculum Vitae

Geert Koene werd op 21 juli 1977 geboren te Heerlen. In 1995 behaalde hij het VWO diploma aan het Bernardinus College te Heerlen. In datzelfde jaar begon hij aan de studie werktuigbouwkunde aan de Universiteit van Twente. In 1996 maakte hij de overstap naar de Universiteit van Amsterdam waar hij Medische Biologie studeerde. Tijdens zijn opleiding deed hij een stage bij de vakgroep Medische Microbiologie van het Academisch Medisch Centrum (AMC) in Amsterdam onder begeleiding van Dr. S.A. Zaat. Een tweede stage liep hij bij de afdeling Moleculaire Celbiologie en Genetica van het Academisch Ziekenhuis Maastricht (AZM) onder begeleiding van Dr. V.L.J.L. Thijssen en Dr. G.J.J.M. van Eys. In augustus 2000 behaalde Geert het doctoraal examen. Aansluitend begon hij met zijn promotieonderzoek op de afdeling Pathologie van het Universitair Medisch Centrum (UMC) Utrecht onder begeleiding van Dr. M.G.J. Tilanus en promotoren Prof. Dr. P.J. Slootweg en Prof. Dr. J.G. van den Tweel. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

List of Abbreviations

ABC	ATP-Binding Cassette
APCs	Antigen Presenting Cells
APM	Antigen-Processing Machinery
ATP	Adenosine 5'-triphosphate
BH	Bleomycin Hydrolase
BSA	Bovine Serum Albumin
β2m	β2 microglobulin
CGH	Comparative Genomic Hybridization
CTL	Cytotoxic T Lymphocytes
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
ERAPI	Endoplasmic Reticulum Aminopeptidase I
ERp57	Endoplasmic Reticulum Protein 57
FISH	Fluorescence *in situ* Hybridization
HLA	Human Leukocyte Antigen
HNSCC	Head and Neck Squamous Cell Carcinoma
HSD	Haring Sperm DNA
IFN-γ	Interferon-γ
KIR	Killer Cell Ig-like Receptor
LAP	Leucine Aminopeptidase
LCM	Laser Catapulted Microdissection
LOH	Loss of Heterozygosity
mAb	Monoclonal Antibody
MFI	Mean Fluorescent Intensity
MHC	Major Histocompatibility Complex
NK	Natural Killer
PA28α	Proteasome Activator 28α
PBL	Peripheral Blood Lymphocytes
PBS	Phosphate-Buffered Saline
PSA	Puromycin-Sensitive Aminopeptidase
PSMB	Proteasome Subunit β
SBMA	Sequencing-Based Mutation Analysis
STR	Short Tandem Repeat
TAA	Tumor Associated Antigens
TAP	Transporter Associated with Antigen Processing
TCR	T Cell Receptor
TOP	Thimet Oligopeptidase
TPPII	Tri-Peptidyl Peptidase
Ub	Ubiquitin