Microarray-based gene expression analysis for the investigation of immunologically relevant processes in tumors

Microarray-basierte Genexpressionsanalyse zur Untersuchung immunologisch relevanter Vorgänge in Tumoren

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Preface

Some chapters of this thesis have been published before. At the beginning of such chapters, it is indicated which experiments were done by the author of this thesis, which persons contributed to the publication, and in which journal the work has been published.

1 Introduction

Principles of Cancer Immunotherapy

Fighting a tumor with the body's own weapons is an alluring concept, especially against the background of conventional cancer therapies, which often lack the specificity crucial for a strong efficacy without severe side-effects. The immune system has been traditionally considered to be an effective means in particular against infectious diseases. On the other hand, the idea that it could protect the host from neoplastic disease is not new either and was initially proposed by Paul Ehrlich [1]. Later, this notion was more explicitly formulated as the "cancer immunosurveillance hypothesis" [2-4]. In the following decades this issue was heavily debated, mostly due to the lack of suitable animal models [5] or other experimental evidence which could definitely confirm this hypothesis. Today, however, there is compelling evidence that substantial interactions between tumors and the immune system take place [6, 7] and that immune cells can actually play an important role in the control of malignancy [8, 9].

Despite their recognition by the immune system, tumors obviously find ways to escape immunosurveillance and establish themselves within the body. Various mechanisms by which cancer cells circumvent recognition or elimination by immune cells have been frequently observed: impaired presentation of antigens to T lymphocytes either by loss or downregulation of HLA molecules [10] or by defects in antigen processing [11]; specific loss of targeted tumor antigens [12-14]; loss of natural killer (NK) cell activating or T cell costimulatory ligands [15, 16]; production of immunosuppressive cytokines [17] or T cell inhibitors [18]; inhibition of proinflammatory danger signals [19]; or specific attraction of immunosuppressive regulatory T cells [20].

Even though tumors possess this impressive arsenal of countermeasures, directing immune responses against them is not a hopeless effort. In some cancer patients spontaneous tumor regression occurs, most likely due to a regained responsiveness to immunologic mechanisms [21]. This indicates that even in established tumors the process of immune escape can be reversed. Furthermore, a vast number of clinical studies aiming at the generation of different kinds of tumor immune responses have shown promising results:

Measurable responses could be frequently generated, sometimes followed by real clinical benefit for the patients [8, 9, 22]. However, the difficult task for any immunotherapeutic concept in order to be ultimately successful is to take the adaptability of tumors under selective pressure into account and adequately address each of the various obstacles likely to be present in established tumor environments.

1.1 Immunotherapeutic approaches

1.1.1 Exploiting the innate immune system

If pathogens succeed in invading their host, they are first confronted with defense mechanisms of the innate immune system. These mechanisms can act very fast because they do not require clonal expansion of antigen-specific lymphocytes, which is necessary for an adaptive immune response. In most cases pathogens are already eliminated at this stage. For decades immunological research has mainly focused on the investigation of mechanisms underlying adaptive immune processes. However, triggered by the detection of pathogen-specific receptors on cells of the innate immune system and their ligands, elucidation of the molecular basics of innate immunity has arguably become the most active area of research in immunology over the last years.

1.1.1.1 Toll-like receptors on antigen presenting cells

Toll-like receptors (TLRs) are a very important family of pattern recognition receptors, which recognize specific microbial components and thereby activate the innate immune system. So far 11 mammalian TLRs are known [23], whereof 9 are conserved between the human and mouse. TLR10 is non-functional in the mouse whereas TLR11 is not expressed in humans [24, 25]. Upon binding of the respective specific ligand, TLR signaling via several pathways [26] triggers the expression of various genes that are involved in immune responses, such as inflammatory cytokines and costimulatory molecules on cells of the innate immune system. This activation is essential for the generation of adaptive immune responses. For example, the activation and clonal expansion of antigen-specific naive T lymphocytes requires their interaction with professional antigen presenting cells (APCs) such as dendritic cells (DCs) that have been activated before, often via TLR signaling.

This central role for the establishment of immune responses makes the innate immune system an important target for cancer immunotherapies. Interestingly, experiments by William Coley in the 1890s - now often considered the first specific attempts of anti-cancer vaccination - made use of exactly those principles. After observing a spontaneous remission in a cancer patient that had

acquired a skin infection, he tried to mimic this effect by inoculating other patients with the same organism causing the disease, *Streptococcus pyogenes* [27]. Due to the severe side effects of this live vaccine he later switched to bacterial extracts which still contained the relevant danger signals. We now know that several components of such extracts are specific ligands for different TLRs [28] and therefore may cause an inflammatory response that can lead to adaptive immunity specifically aimed at present tumor antigens.

Today there is just one cancer species, superficial bladder cancer, for which bacterial treatment (with Bacillus Calmette-Guerin (BCG)) leading to immune activation is the established standard therapy [29]. The identification of TLR ligands has brought new momentum to the field by enabling the production of molecularly defined stimulatory agents. In contrast to bacterial surface components, nucleic acids can be more easily produced in drug quality. Bacterial CpG DNA motifs act via TLR9 [28] and have been effectively applied alone [30] or as adjuvants in antigen-specific vaccinations in tumor immunotherapy [31]. Similarly, single-stranded RNA has been shown to activate innate immune processes via TLR7 and TLR8 and can be used for immune activation [32-35]. In addition, artificial synthetic TLR7/8 agonists are a promising alternative [36, 37].

1.1.1.2 Cytokines

An important component of innate immune reactions is the production of various cytokines, which may directly act on tumor cells or regulate further immune processes. Type 1 interferon secretion, mostly by DCs, is one of the first events in the innate immune response after antigen recognition [38]. These cytokines simultaneously act as a differentiation and maturation signal for DCs [39], activate natural killer (NK) cells [40], or directly affect tumor cells by sensitizing them for apoptosis [41] or inhibiting tumor angiogenesis [42]. Similar effects on tumors have been reported for interferon-γ (IFN-γ) [43-45]. In addition, IFN-γ is crucial for the upregulation of MHC class I and II molecules and the necessary antigen processing and presentation pathways, thus enabling effective T cell responses against the tumor [46]. Interferon-α (IFN-α) is therapeutically used against different cancers and is most effective in hematological malignancies [47] but is also used for post-surgical adjuvant therapy in high-risk solid tumor settings [48, 49].

Interleukin-12 (IL-12) is produced by phagocytic cells and DCs upon antigen recognition and has important effects on T cells and NK cells. It is the major cytokine for Th1-cell differentiation, allowing potent IFN-γ production [50]. Dependent on the presence of NK, NKT, and T cells, which IL-12 can drive towards their most active effector functions against tumors, this cytokine has a potent anti-tumor activity [51]. IL-12 has been rarely used alone in clinical trials due to considerable toxicity [52]. In several adjuvant settings in connection with peptide-based vaccinations of melanoma settings, IL-12 has shown some encouraging results [53, 54].

Granulocyte-macrophage colony stimulating factor (GM-CSF) is mainly produced by T cells but has important effects on DC maturation necessary for the induction of potent adaptive immune responses [55]. Promising therapeutic strategies comprise vaccines of tumor cells engineered to secrete GM-CSF [56, 57], adjuvant ex vivo maturation of DCs which are then loaded with antigen and transferred to the patient [58], or using GM-CSF directly with the vaccine *in vivo* [59].

Interleukin-2 (IL-2) is a cytokine which is not directly related to the innate immune system but is frequently used in tumor immunotherapy. It is the most important T cell proliferation factor and might therefore act by enhancing inadequate T cell responses against tumors [60]. Il-2 has been either used alone [61], with synthetic peptides [62], or in connection with adoptive T cell transfer [63].

1.1.1.3 Natural Killer cells and γδ T cells

Natural killer (NK) cells belong to innate immunity because their receptors are encoded by germline genes that do not require somatic recombination. Functionally, however, they more closely resemble T cells than any other leukocyte of the innate immune system, because they possess the same killing mechanism as cytotoxic T lymphocytes (CTL) and secrete IFN-γ like CTL and Th1 helper T cells [64]. NK cells express an immense repertoire of activating and inhibitory receptors which all contribute to the resulting signal that decides about whether the cell is activated or not by the target cell. According to the classic "missing self recognition" concept [65], NK cells can be activated by loss of MHC class I molecule expression on target cells, which normally bind to inhibitory NK receptors. Consequently, NK cell mediated killing of MHC class I

negative tumor cells has been frequently observed [66]. More recently, research on NK cell activation has shifted its focus more to activating receptors, mainly to NKG2D [67], whose ligands are absent from normal cells but can be induced by stress like infections, but also tumorigenesis [68]. Expression of NKG2D ligands on MHC class I positive tumor cells can overcome NK cell inhibition and lead to tumor lysis [69]. Due to its novelty, only few approaches in mice are known making use of the NKG2D system and its ligands for immunotherapy [70, 71].
The human NKG2D ligands MICA and MICB can also be recognized by the T cell receptor (TCR) of a subset of γδ T cells mostly located within intestinal epithelia [72, 73]. In contrast to "classical" αβ T cells which account for the vast majority of all T cells and recognize short peptides bound to MHC molecules, γδ T cells display a very restricted TCR repertoire and recognize a variety of possible antigens independent of MHC. They exhibit functions similar to αβ T cells and NK cells such as cytokine production and killing [74] and might even play a role as professional APCs [75]. γδ T cells have been found at relatively high proportion among tumor-infiltrating lymphocytes (TILs) of various origins [76] and several mouse models indicate that they likely play a role in tumor immunosurveillance [77-79]. The only well-characterized tumor ligands for human γδ T cells are the NKG2D ligands MICA and MICB. Since γδ T cells also express NKG2D, activation may occur via both, TCR and NKG2D binding [80]. Therapeutic applications of γδ T cells against cancer have been so far restricted to rather unspecific stimulations *in vivo* using aminobisphosphonates in combination with IL-2 [81, 82].

1.1.2 Antibody-based therapies

B lymphocytes are the fundamental cells of the adaptive humoral immune response. Their main function is the production and secretion of highly target-specific antibodies. The extremely diverse repertoire of specificities that B cells are able to generate [83] can be exploited for various applications. Following the invention of stable hybridoma cell lines that are able to secrete virtually unlimited amounts of specific monoclonal antibodies (mAbs) [84], such antibodies have become one of the most widely-used tools in life sciences.
Over the last years, various mAbs have been established for therapeutical use in humans. mAbs were first used for cancer therapy more than 20 years ago

[85], but showed only limited efficacy in the beginning. This was due to the immunogenicity of unmodified mouse antibodies used in humans, leading to their fast elimination by antibody responses directed against the mAb in patients. Equally important, mouse mAbs are not able to efficiently recruit effector mechanisms in the patients that can kill the target cells upon antibody binding. Therefore, humanized antibodies were developed, which contained only the mouse parts necessary for specificity engineered into a human antibody [86, 87]. Activation of appropriate effector mechanisms is an important issue for all antibody therapies. Even though some mAbs might act autonomously by blocking signaling pathways or inducing apoptosis [8], they rely in most cases on help from host systems in order to be effective. Antibodies bound to target cells can activate the complement system, a collection of plasma proteins which are ultimately able to cause inflammation, recruit phagocytes, or directly lyse pathogens or other cells covered by antigen-antibody complexes [88]. Furthermore, binding of the Fc part of antibodies by Fc-γ receptors on effector cells [89] is a prerequisite for antibody-dependent cell-mediated cytotoxicity (ADCC), a mechanism mainly mediated by NK cells and macrophages and likely a dominant component of anti-tumor activity of mAbs [90].

The first mAbs approved by the U.S. Food and Drug Administration (FDA) for treatment of cancer were Rituxan [91] (1997) against CD20 on B cells in Non-Hodgkin Lymphoma and Herceptin [92] (1998) against the HER2/neu tyrosine kinase receptor often upregulated in breast cancer. Both antibodies have been shown to improve the overall survival when added to standard chemotherapy in randomized trials [93, 94]. A promising modification to increase the effector function of mAbs is to arm them with toxins [95] or radionuclides [96] coupled to their Fc parts. The idea behind this is to specifically guide cell-damaging agents to the desired target cells. Another option for the specific recruitment or activation of effector cells at the tumor site are bispecific antibodies containing a specificity for a tumor antigen and one for effector cell markers or costimulatory receptors, such as CD3 or CD28 on T cells [97, 98].

In the meantime, various antibodies for cancer therapy have obtained FDA approval and a lot more are in clinical trials. They are mostly used in combination with other treatments like standard chemotherapy and frequently

show clinical benefit. Thus, immunotherapy by passive administration of antibodies has become a clinical reality [99].

A different approach to utilize the therapeutic potential of antibodies is their *in vivo* induction by vaccination. For example, anti-idiotypic antibodies against B cell lymphomas have been generated by pulsing DCs with the unique idiotypic immunoglobulin (Ig) of the lymphoma to induce T helper cells followed by boosting with Ig coupled to keyhole limpet hemocyanin (KLH) [100]. This vaccination resulted in prolonged remissions but it is difficult to assess the exact role of the antibodies in this setting. Thus, while active immunization approaches are still in early experimental stages, the field of antibody-mediated cancer therapy will likely continue to be dominated by passive administration of mAbs specifically designed for selected tumor antigens.

1.1.3 Cellular adaptive immunotherapy

T lymphocytes are the cells mediating cellular adaptive immunity. αβ T cell receptors (TCRs), present on most T cells, recognize short peptides bound to major histocompatibility complex (MHC) proteins [101]. T cells expressing the CD8 coreceptor (CD8+ T cells) recognize peptides on MHC class I (MHC-I) molecules mainly derived from intracellular source proteins whereas CD4+ T cells recognize peptides on MHC class II (MHC-II) molecules. MHC-II expression under normal conditions is restricted to immune cells [102], mainly APCs specialized in taking up extracellular antigens, the main source of MHC-II peptides. Therefore, T cell based tumor immunology has classically focused on CD8+ positive cytotoxic T lymphocytes (CTL) which should be able to kill tumor cells upon recognition of peptides derived from tumor associated proteins.

To mediate anti-tumor-activity, T cells must first be activated by dendritic cells (DCs), the most effective APCs for T cell priming [103]. Therefore, appropriate activation of the innate immune system as discussed in chapter 1.1.1 is an indispensable prerequisite for successful T cell immunotherapies especially because non-activated DCs might generate tolerance against the vaccine [104]. Properly activated and differentiated effector CTLs may then be able to gain access to the tumor and either directly lyse tumor cells via the perforin and/or Fas pathway [105]. Furthermore, CTLs may produce cytokines such as IFN-γ

that can arrest the proliferation of malignant cells or inhibit tumor angiogenesis [45].

Like for antibodies, the selection of appropriate tumor associated antigens (TAAs) is the crucial first decision to be made. Since this selection process is the main objective of this thesis, it will be addressed in detail in the following chapter 1.2. An alternative to defining specific antigens are vaccines based on either autologous whole tumor cells [106, 107], allogeneic tumor cell lines [108], or isolated components of tumors, like total RNA [109] or heat shock protein-peptide complexes [110]. Such vaccines are mostly applied in combination with DCs in order to enable T cell priming [111] or tumor cells are being directly fused with DCs [112]. Such undefined approaches can be easily carried out in a patient-individual way, thereby including all individual antigens that may have arisen during tumorigenesis in this patient. Other advantages are that the vaccine can be applied independent of the HLA type, may include T helper cell epitopes, and no extensive analytical work has to be done to enable vaccination. However, these advantages are faced by some serious drawbacks: The undefined nature of the antigens makes a controlled monitoring of tumor-reactive T cell populations virtually impossible and therefore prevents a step-wise dissection of processes on the way to tumor regression or failure of the therapy. Moreover, important TAAs might be present in the vaccine at concentrations too low to induce a response, or the majority of irrelevant components might even induce autoimmune reactions in the potentially highly immunogenic environment established by the vaccination.

If specific antigens are to be used, there are several possible application forms, which again have to take proper DC activation into account. DCs can be either isolated and loaded with antigen ex vivo or the antigen can be designed to reach and activate DCs *in vivo*. MHC binding peptides have been frequently used for vaccinations either directly with or without adjuvants [59, 113] or loaded on autologous DCs before [59, 114, 115]. Peptide vaccinations have some limitations due to the restriction of peptides to their specific HLA type, the limited number of known TAA peptides, or the possibly short half-life of MHC-peptide complexes on DCs after loading, which may cause dissociation of a large proportion of MHC-peptide complexes already on the way to draining lymph nodes. The latter point has been addressed by various attempts to modify natural MHC ligands in order increase their affinity to MHC proteins [116,

117]. It is, however, questionable whether T cells generated to recognize such peptides are still able to do so for the natural form occurring on the tumor [118].
Recombinant proteins may be used as an alternative to peptides [119-121]. Depending on efficient processing, they have the potential to deliver T cell epitopes for various HLA alleles, including MHC-II. Unfortunately, they are extremely difficult to produce in clinical grade. Immunizations using recombinant virus or naked DNA are an alternative [122-125]. Do to their faster degradation, mRNA based vaccines can be more easily controlled and do not have the risk of undesired genomic integration like DNA constructs. Therefore, mRNA has been frequently used for DC transfection [126-128] and might be even effective by direct *in vivo* application [129].
Despite the attractiveness of active *in vivo* induction of anti-tumor T cell reactions, adoptive T cell transfer has so far been the more successful approach [22]. While allogeneic T cell transfer has the advantage of mediating an associated graft-versus-tumor (GvT) effect by reactions against minor histocompatibility antigens [130], this is accompanied by the risk of severe graft-versus-host disease (GvHD) in the patient [131]. Therefore, autologous approaches are often employed, such as the isolation of tumor-infiltrating lymphocytes (TILs) followed by an *in vitro* expansion of tumor-reactive TILs and reinfusion into the patient, often after lymphodepletion [13, 63]. Objective clinical response rates of up to 50% have been reported for such studies [132]. In several studies it has been tried to genetically manipulate the TCR in order to increase its affinity for the TAA and adoptively transfer the resulting clones [133-135]. Alternatively, chimeric receptors comprised of an antibody recognition domain fused to signaling domains of the TCR [136] or costimulatory molecules [137] have been used.
Altogether, due to their specificity directly coupled to potent effector functions, T cells are an attractive means for tumor immunotherapy. Their success in reliably fighting established tumor masses, however, will largely depend on the selection of appropriate tumor antigens and finding the right stimulation procedures to maintain properly differentiated effector and memory cells at higher numbers over longer periods.

1.2 Tumor associated antigens

The existence of structures distinguishing tumors from normal self is the indispensable basis for cancer immunotherapy. Knowledge about which of such tumor associated antigens (TAAs) are expressed by the specific tumor, how they may arise, and what the advantages and restrictions are for different types of TAAs, is an important prerequisite for the design of suitable therapeutic approaches.

1.2.1 A classification of TAAs

Every alteration occurring during tumorigenesis at the protein level can potentially cause recognition by T cells, provided that the altered protein can be processed and presented by MHC molecules. Therefore, it is not surprising that virtually all imaginable quantitative and qualitative changes have already led to observable T cell responses. Based on their origin or the specific process, by which such tumor epitopes are generated, several systems to classify TAAs are possible. The classification depicted in Table 1.2.1.1 follows a common scheme as applied by Novellino et al. [138].

Table 1.2.1.1. A selection of human tumor antigens recognized by T cells. Adapted from Novellino et al. [138].

Class of antigen	Subclass	Examples of antigens	References
Germ cell / Cancer-testis antigens		MAGE-A1	[139-141]
		MAGE-A2	[142]
		MAGE-A3	[143, 144]
		NY-ESO-1 (CTAG1B)	[145, 146]
		SSX-2	[147-149]
Differentiation antigens	*expressed in melanocytes*	Melan-A / MART-1	[150-152]
		tyrosinase	[153, 154]
		gp100	[155, 156]
	expressed in embryonic tissue	CEA	[157-159]
	expressed in prostate	PSA	[160, 161]
Widely occurring overexpressed antigens		adipophilin	[162]
		HER-2/neu	[163-165]
		c-met oncogene	[166]
		MUC1	[167, 168]
		survivin	[169-171]
		WT1	[172, 173]

Tumor specific antigens	*point mutations*	β-Catenin	[174]
		CDK4	[175]
		ras	[176-178]
	gene translocation and fusion	bcr-abl	[179-182]
	alternative mRNA splicing	TRP-2 (DCT)	[183]
	translation from an alternative ORF	BING-4 (WDR46)	[184]
	translational frameshift	TGFBR2	[185, 186]
		OGT	[187]
	translated intron	TRP-2 (DCT)	[188]
	posttranslational amino acid modification	tyrosinase	[189]
	*protein splicing**	FGF5	[190]
		gp100	[191]
Oncoviral proteins		HPV16 E7 protein	[192, 193]

* Protein splicing itself is most likely not tumor specific. Recognition of such epitopes by anti-tumor CTLs is probably rather due to the overexpression of FGF5 and gp100 in the respective tumors.

Since the identification of the first gene encoding a T cell epitope [139, 194] recognized on human tumor cells, there have been intensive efforts to identify such possible targets for a variety of cancers. This led to the identification of a considerable number of TAAs [138] generated by various mechanisms (Table 1.2.1.1). Despite this success, the number of identified TAAs is still far away from being sufficient for comprehensive immunotherapeutical approaches based on molecularly defined antigens. One reason is the strong bias towards melanoma associated antigens, one of the most widely used model cancer in tumor immunology. A second bias is towards epitopes restricted by HLA-A*02. Even though this is the most frequent HLA class I allele in the caucasian population, HLA-A*02 epitopes are useless for more than 50% of the patients. In consequence the so far known TAAs are restricted to very few cancer species and HLA alleles.

Concerning the different classes of antigens listed in Table 1.2.1.1, there are profound differences with respect to their quality for immunotherapy.

1.2.1.1 Cancer-testis antigens

This group of antigens has its name from their observed expression pattern: among healthy tissues, they are exclusively expressed in spermatocytes of testis and sometimes in placenta [195]. Because these cells do not express

HLA molecules [196], T cells in healthy individuals are normally not tolerized against those self-antigens. Upon tumorigenesis, they are transcriptionally activated in certain tumors and may provide tumor associated targets for T cells. Since cancer-testis antigens have no known physiological function in germ cells or tumors, cancer cells are likely able to escape from immune responses by downregulation of antigen expression [197].

1.2.1.2 Differentiation antigens

These TAAs are shared between the tumor and the normal tissue from which the tumor arose. Most of the identified TAAs of this class belong to the melanoma/melanocyte group. Melanocytes are specialized in melanin biosynthesis and many enzymes needed for this are therefore highly specific for these cells. Expression of such melanocyte-specific genes in healthy individuals may lead to T cell tolerance against these antigens, which has to be broken to achieve an immune response in anti-melanoma vaccinations. T cells against differentiation antigens may lead to autoimmunity against the corresponding normal tissue. While the prostate is often completely removed in the case of prostate cancer, making autoimmunity against prostate antigens unlikely, melanoma vaccinations are often accompanied by vitiligo, due to melanocyte destruction [198]. However, such side effects against dispensable normal tissues can be justified in the face of potential therapeutic benefit.

1.2.1.3 Widely occurring overexpressed antigens

This class of antigens is expressed in many normal tissues, albeit at generally lower levels compared with tumors. Even though one might expect a profound T cell tolerance against such antigens, this is actually the TAA class with the highest number of members. Several mechanisms might explain, why reactive T cells against epitopes from such antigens exist. The expression levels of such antigens in the thymus or peripheral organs may be too low to lead to epitope densities sufficient for overcoming ignorance. Alternatively, altered antigen processing in tumors may cause the generation of MHC-bound peptides not present in other tissues.

The advantage of these TAAs is their potential expression in different types of cancer, because tumorigenesis is likely to require general regulatory

mechanisms leading to comparable expression changes independent of the specific tissue [199]. This may implicate as an additional advantage the dependency of the tumor on the respective TAA, making loss of expression rather difficult. Unfortunately, however, these advantages are opposed by potentially severe drawbacks on the T cell side. The chance of breaking tolerance against widely expressed antigens may be low, and in case of successful establishment of a T cell response, one has to worry about autoimmunity against vital organs. Nevertheless, overexpressed TAAs have been used in several clinical trials showing specific T cell induction *in vivo* and low risks of severe autoimmune reactions [200-203].

1.2.1.4 Specific antigens

Truly tumor specific T cell antigens can consist of peptides with either an amino acid sequence or posttranslational modifications not occurring in the normal proteome. Whereas reports on T cell recognition of posttranslational modifications like altered glycosylation [204] or altered amino acid side chains [189] are rare, many examples are known for mechanisms leading to unique peptide sequences recognized by T cells in tumors.

Chromosomal translocations may lead to new epitopes at the fusion site. Such examples are mostly known for hematopoietic malignancies, especially chronic myelogenous leukemia (CML) [179-182]. Other frequently observed alterations at the genomic DNA level are point mutations [174-178]. At the mRNA level, unusual splicing may generate tumor specific antigens [183]. For the next step on the way to the MHC ligand, various abnormal translation events have been reported [184-188]. Of note in this context is the possibility that unusual start codons like CUG might lead to the generation of unexpected antigens, even though T cell recognition of such epitopes has only been described for mice so far [205, 206]. Recently, epitopes have been detected which are based on posttranslational splicing events within single proteins [190, 191]. The mechanism underlying this phenomenon is likely to involve proteasomal processing [191]. It is, however, not clear whether splicing itself has anything to do with the tumor. Most likely, tumor association of such epitopes is rather due to the overexpression of the respective protein in the tumor.

Tumor specific antigens are the ideal targets for T cell based cancer immunotherapy, because there is no risk of autoimmunity, and tolerance

against these antigens should be less profound than for shared antigens. The best antigens among them are mutations which are essential for the tumor, such as in cell cycle regulators [175] because the tumor cannot easily get rid of them. The disadvantage of this class of TAAs, however, is their probably rather patient-specific occurrence. Even though some underlying mechanisms, like chromosomal translocations in leukemia, are a general hallmark of the respective cancer and can be easily screened for, both does most likely not apply to the majority of mechanisms listed here. In addition, one single defined mutation will generally lead to new epitopes only for a low number of HLA alleles, restricting its therapeutic potential to few patients. Against this background, it would be highly desirable to know at least some tumor specific antigens for each patient in order to be able to design an individualized vaccine. Unfortunately, with the current analytical technology, a feasible strategy to reliably detect even only few of such potential epitopes for a given patient within a reasonable time and with reasonable efforts is far out of reach yet.

1.2.2 Strategies for the identification of tumor associated T cell epitopes

1.2.2.1 The classical approach: Starting with T cells recognizing the tumor

The group of Thierry Boon used an expression cloning approach to identify the first gene encoding an antigen recognized by a CTL on a human tumor - MAGE-A1 [194]. Cytotoxic T cells were isolated from a patient and used to screen expression libraries prepared from the recognized tumor cell, leading to the relevant gene. The gene containing the epitope was further narrowed down by truncation to a 0.3 kb region. The last step to the epitope was done by “epitope mapping”, a frequently used method since these days. The primary sequence is represented by many adjacent or partially overlapping small peptides of about 15 amino acids in length and each of the peptides is analyzed for its ability to mediate CTL response if loaded on cells expressing the respective MHC molecule. When a 15-mer peptide is recognized, all possible nonamer peptides included in the sequence are tested again. Thus, the HLA-A*01-restricted epitope (EADPTGHSY) was identified [139].

Another approach also starting with T cells was pioneered by the group of Slingluff [207]. Using the method described by Falk et al. [208], they isolated MHC-bound peptides from a melanoma cell line, which was recognized by melanoma-specific CTL lines from five different melanoma patients. After separation by HPLC, fractions were tested for their ability to reconstitute epitopes for two of the five CTLs after loading on T2 cells. Three peptides coeluting with the cytotoxic activity were sequenced by tandem mass spectrometry, and finally one of them, (YLEPGPVTA), was shown to be recognized by all five melanoma-specific CTLs.

1.2.2.2 Reverse immunology: Starting with known tumor antigens

The existence of HLA-allele-specific peptide motifs [208] is the basis for this approach. Characteristic lengths and sequence properties of peptides bound to each specific HLA allele restrict the otherwise unmanageable number of possible ligands derived from a given protein considerably and enable their prediction. Therefore, if a certain tumor antigen in connection with a specific HLA allele (with a known binding motif) is of interest, ligands that most likely bind to the HLA molecule can be predicted from the protein sequence. Thus, this approach does not rely on pre-existing T cells. It is, however, necessary to raise T cells against the predicted epitope in order to confirm actual recognition. The final proof should always come from a reaction of those T cells against a tumor cell line expressing the antigen in order to confirm its natural processing.
Several programs offer epitope predictions for various HLA alleles, as for example BIMAS/HLA_BIND [209] or SYFPEITHI [210]. Predictions should also take into account proteasomal processing [211] and transport into the endoplasmic reticulum [212] of potential epitope precursors. A recent approach has combined all three steps into one integrated prediction algorithm [213]. As an important additional step to verify processing and presentation in actual tumor samples, predictions can be used to detect the peptide directly by mass spectrometry [214]. This approach has led to the identification and confirmation of an epitope from MAGE-A1 on HLA-A*02 [141].

1.2.2.3 The third way: Starting with MHC ligands - definition of novel TAAs by mass spectrometry and gene expression analysis

This approach, pioneered by Weinschenk et al. [215] aims at the identification of as many HLA ligands as possible from primary human tumors. From these data alone, it is not clear whether any of the peptides might be a candidate TAA. Therefore, in an additional step, whole-genome mRNA expression profiling is performed using oligonucleotide microarrays. Comparative analysis of the primary tumor, autologous corresponding normal tissue, and a panel of normal tissues pooled from several healthy individuals enables a relatively quick assessment of expression profiles for the genes underlying the identified peptides. Candidate TAA epitopes, characterized by a favorable expression profile, i.e. high expression in the tumor and low or undetectable expression in normal tissues, have the advantage that they are known to be processed and presented on real tumors in their natural environment and can be verified by the generation of T cells reacting against the peptide bound to the respective HLA allele. Two candidates identified by this approach have been already confirmed as TAA epitopes: adipophilin [162] and met proto-oncogene [166]. An additional advantage is the applicability in a patient-individual way, because all analyses can be carried out within few weeks and may ultimately lead to a set of several candidate TAAs fitting specifically to the tumor and therefore likely well-suited for an individualized vaccination. Altogether, this approach may lead to the identification of considerable numbers of new overexpressed TAAs which may be either patient-individual or may be of use in a more general way for cancer immunotherapy.

While HLA ligands are in the center of this approach, they by themselves do not necessarily convey information about their tumor association. In fact, the majority of peptides identified from tumors originates from normal self-proteins that are irrelevant for immunotherapy. The definition of overexpressed TAAs requires some kind of quantitative information. Ideally, this information should be direct quantitative information of HLA ligand densities on the tumor and normal tissues, since this is the relevant parameter for T cell reactions [216-218]. While methods exist to directly perform such quantitative comparisons [219], their routine application is still difficult and certainly cannot address ligand expression in different organs in order to obtain information about the grade of tumor association. Therefore, mRNA or protein levels have to be used as

surrogate parameters to define overexpression. The standard tools for protein quantification are based on monoclonal antibodies. Because such antibodies are not available for the majority of proteins, comprehensive quantifications including all source proteins of identified HLA ligands are impossible at present. Innovative solutions may arise in the future from the emerging field of protein microarray technology [220].

For the time being, mRNA expression is the only relevant parameter that can be quantitatively assessed in a comprehensive and routine setting by DNA microarrays. Since their invention in the mid 1990s [221], technology has rapidly developed and microarrays have now become a routine analytical tool accessible for many labs. Besides the still common original approach of printing of cDNAs on glass slides, oligonucleotide arrays are another important class of DNA microarrays [222]. During production of these arrays developed by Affymetrix, oligonucleotides are synthesized in situ on glass slides in a highly parallel fashion using photosensitive protection groups [223]. While cDNA arrays are more flexible, because each desired cDNA can be spotted, they bear the risk of major quality changes or errors due to confusion or contamination of cDNA samples during production. Considering the availability of commercial whole-genome oligonucleotide arrays for the most widely studied organisms, especially Affymetrix arrays are becoming more and more the gold standard. An advantage of such high-density oligonucleotide microarrays is their system of addressing each gene by several oligonucleotides (in general 11 25-mers plus 11 mismatch controls) allowing for a certain level of statistical analysis for each gene. Furthermore, the Affymetrix system uses an integrated instrumentation approach, which is not as flexible as cDNA array systems but is likely to increase reproducibility [224] and has been shown to introduce less lab-to-lab variance compared to other systems [225]. The reliability and quality of this technology is also illustrated by the first FDA approval ever granted to a DNA microarray test for clinical diagnostics in 2004, which is manufactured by Affymetrix (http://www.fda.gov/bbs/topics/news/2004/new01149.html). Due to these advantages and despite their relatively high costs, Affymetrix arrays have been used for all microarray experiments described in this thesis.

An important question is whether mRNA overexpression actually indicates higher presentation levels of HLA ligands from the respective protein. Recently, studies comparing mRNA and protein levels for larger number of genes have

found only weak correlations [226, 227]. However, this need not be true to the same extent for mRNA and HLA ligands. It is now widely believed that a large proportion of HLA ligands is derived from defective ribosomal products (DRiPs) [228], aberrantly translated proteins which are then degraded by the proteasome [229, 230]. Via this mechanism translation and thus mRNA levels might be more closely coupled to antigen processing and presentation than to steady state levels of intact proteins. Furthermore, protein levels are likely not a good indicator of epitope generation because they do not reflect turnover. Thus, mRNA might in fact be the best available surrogate marker for HLA ligand levels. However, it is unclear how far correlations are perturbed by the various processing steps lying in between mRNA and final peptide on the cell surface. Altogether, using mRNA expression data to define overexpressed TAAs seems to be justified by successful examples for epitopes identified by this means [162, 166, 231].

Assessing the feasibility of this approach at a larger scale, as shown in chapter 2.1, and exploring the fundamentals of its extension to tumor species other than renal cell carcinoma, as shown in chapter 2.2, were major aims of this thesis.

1.3 Obstacles and opportunities on the way to an effective anti-tumor response

While the identification of suitable TAAs is a necessary prerequisite for any molecularly defined tumor immunotherapy, it is clearly not sufficient for achieving an effective anti-tumor response. Therefore, much effort has been put into the development of strategies to improve priming of naive T cells against TAAs in therapeutical settings by appropriate DC differentiation and maturation signals such as different cytokines and TLR ligands [58]. Nevertheless, objective clinical response rates of cancer vaccine trials have been clearly below 10% [22, 232]. This is a clear indication that appropriate therapeutical settings have not been found yet.

The focus so far has mostly been on generating CD8+ CTL responses but not so much on maintaining them. It is, however, becoming more and more evident, that CD4+ T cell help is crucial for the maintenance of fully functional CD8+ T cell immunity, including memory [233-235]. Therefore, efficacy of cancer vaccines could likely be improved if they were able to induce CD4+ T cell responses. The identification of more TAAs presented in the context of HLA class II might thus be a promising approach to follow. The work presented in chapter 2.3.1 of this thesis contributes to this aim by providing evidence for a novel mechanism for presentation of intracellular antigens on MHC-II molecules on tumor cells. This might be a way for HLA class II positive tumors to present TAAs to CD4+ helper T cells.

Immunologists had obviously to be aware of tumor escape from immune recognition since tumor immunosurveillance had been hypothesized [236]. For a long time, it has been thought that escape was mostly passive, e.g. by downregulation of antigen presentation, and that immune cells could simply not reach sufficient activation due to the lack of costimulatory signals and cytokines. Such issues have been comprehensively addressed by vaccines targeting several TAAs and supplementing activators of costimulation and cytokines without achieving pronounced efficacy in general. However, more and more evidence is emerging for tumor mechanisms that actively target immune cells in order to induce suppression or recruit them for the tumor's own benefit [237, 238]. Such mechanisms include the production of immunosuppressive

cytokines, like TGF-β, IL-10, VEGF, IL-6, or M-CSF, which shift the cytokine balance to a pattern that inhibit normal differentiation and maturation of DCs and generating phenotypes that may induce regulatory T (T_{Reg}) cells [238]. Furthermore, tumors may induce the expression of co-inhibitory ligands such as B7-H1 on DCs [239], which may increase T_{Reg} activation [240] and lead to apoptosis on effector T cells [241]. On the other hand, T_{Reg} cells recruited to the tumor and further stimulated by the mechanisms mentioned above, can act back on APCs e.g. by inducing IDO expression in DCs [20], thereby further shifting the balance from immunogenic to immunosuppressive APCs. By the time vaccination is performed, mostly in late stage tumors, such induced suppressor cells may have already spread to draining lymph nodes, inhibiting the induction of effective responses by active immunization even after surgical removal of the tumor.

Against this background, it is not surprising that the most successful approach for T cell immunotherapy so far has been passive adoptive transfer of *in vitro* generated effector cells after lymphodepletion [22]. This method is likely to weaken suppressive mechanisms and their remaining activity may just be overwhelmed by the vast numbers of effector T cells necessary to achieve an effective response. The observation that even in this setting lymphodepletion is necessary for at least partial success illustrates how powerful immunosuppressive mechanisms might be in cancer patients - or how strictly T cell homeostasis is regulated. However, increasing research efforts in this field will likely lead to treatment options that may effectively target such novel immunosuppressive mechanisms [238].

Since Edward Jenner's pioneering vaccinations, active immunotherapy has become a success story enabling effective protection against various pathogens by smart and well-tolerated strategies. Against this background and despite the rather discouraging present situation with respect to active tumor immunization, it clearly cannot be the ultimate goal of immunologists to fight cancer with brute force passive approaches like adoptive T cell transfer, which will most likely never be applicable to larger patient numbers. Very recent research has come up with novel explanations of why tumors are so difficult to attack with the immune system. If solutions to overcome such suppressive mechanisms are found, the hope is justified that an efficacious active cancer immunotherapy will ultimately become reality.

1.4 Aims of the thesis

At the beginning of this thesis, the proof-of-principle for a patient-individual anti-tumor vaccination approach had been established by Toni Weinschenk [215]. In a renal cell carcinoma model, HLA ligand identification of the tumor was combined with gene expression analysis of tumor and autologous normal kidney in order to identify overexpressed tumor associated antigens (TAAs). In this approach, assessment of gene expression in other normal tissues had been sporadically performed by quantitative real-time RT-PCR (qPCR).

The first aim of this thesis was the extension of this existing strategy to rapidly define TAAs based on identified HLA ligands for a larger number of renal cell carcinoma patients. Such patient-individual vaccination candidates were required for a clinical trial. In order to achieve this goal, gene expression analysis for normal tissues could no longer be performed by time-consuming qPCR for each candidate gene. Therefore an in-house whole-genome microarray expression database had to be established which enabled the fast screening of known or newly identified HLA ligands for their tumor association in individual patients. The results of an analysis of several patients are shown in chapter 2.1.

The second aim was to establish similar approaches for other tumor species. Colon adenocarcinoma is a technically more challenging tumor species for gene expression analysis, because tumor and normal tissue are quite heterogeneous. Therefore, laser microdissection was used to isolate specifically the cells of interest, yielding only minute amounts of RNA, sometimes of impaired integrity. Therefore, some technical issues had to be solved, regarding RNA quality and necessary amplification procedures. Finally, gene expression analysis could be successfully applied to define candidate TAAs for colon carcinoma. These results are shown in chapter 2.2.

The third aim was to assess the potential of comprehensive gene expression analysis for the exploration of more complex immunological processes relevant to tumor immunology. Chapter 2.3 shows that autophagy is a mechanism that can lead to MHC class II presentation of intracellular antigens on tumor cells and how gene expression analysis can contribute to the elucidation of such a process, which might be an important basis for the definition of CD4+ T helper cell TAAs in the future.

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2 Results and Discussion

2.1 General and patient-individual tumor antigens in renal cell carcinoma

2.1.1 Lessons to be learned from primary renal cell carcinomas: Novel tumor antigens and HLA ligands for immunotherapy

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The author of this thesis performed all gene expression experiments and contributed to the evaluation of mass spectra from several RCC samples.

2.1.1.1 Abstract

The lack of sufficient well-defined tumor-associated antigens is still a drawback on the way to a cytotoxic T lymphocyte-based immunotherapy of renal cell carcinoma (RCC). We are trying to define larger numbers of such targets by a combined approach involving HLA ligand characterization by mass spectrometry and gene expression profiling by oligonucleotide microarrays. Here we present the results of a large-scale analysis of 13 RCC specimens. We were able to identify more than 700 peptides, mostly from self proteins without any evident tumor association. However, some HLA ligands derived from previously known tumor antigens in RCC. In addition, gene expression profiling of tumors and a set of healthy tissues revealed novel candidate RCC-associated antigens. For several of them we were able to characterize HLA

ligands after extraction from tumor tissue. Apart from universal RCC antigens, some proteins seem to be appropriate candidates in individual patients only. This underlines the advantage of a personalized therapeutic approach. Further analyses will contribute additional HLA ligands to this repertoire of universal as well as patient-individual tumor antigens.

2.1.1.2 Introduction

Metastatic renal cell carcinoma (RCC) remains a disease with a fatal prognosis. In 2004 more than 35,000 new cases and more than 12,000 cancer-related deaths were estimated in the US [1]. If metastasis is diagnosed, the one year survival rate decreases to approximately 60%. This underlines the dissatisfactory therapeutic situation. Currently numerous new therapeutical approaches are under investigation. The known, albeit rare, phenomenon of spontaneous regression of metastasis in RCC patients [2] and the existence of tumor-reacting and -infiltrating cytotoxic T lymphocytes suggests that RCC is an immunogenic tumor. Several immunological concepts of therapy have been proposed and several tumor-associated antigens (TAA) defined for RCC in the past. The aim of our investigations was to identify HLA class I-presented peptides characteristic for the tumor *in vivo*. These peptides, processed from proteins characteristically expressed in the malignancy, may serve as targets for a vaccination- induced cytotoxic T lymphocyte (CTL) response against the tumor. To achieve this goal, we performed mass spectrometry (LC/MS)-based peptide sequencing as well as patient-individual microarray gene expression profiling (Figure 2.1.1.1) with surgically resected RCCs. This led to the generation of a data set providing information on the one hand about the sequences of approximately 100 HLA-presented peptides for each tumor specimen of appropriate mass, on the other hand about the level of expression for approximately 14,000 particular genes in every tumor. Overexpressed genes were identified in individual tumors in comparison to a broad set of healthy tissues, covering most human organs. Extensively upregulated genes are expected to give rise to tumor-associated proteins and peptides, which should provide targets for specific CTL recognition of the tumor [3]. We consider such peptides suitable for vaccination. Combining both analytical tools, peptide analysis and gene expression profiling, we are able to identify such potential

CTL targets in individual malignancies, which might ultimately find their way into clinical applications.

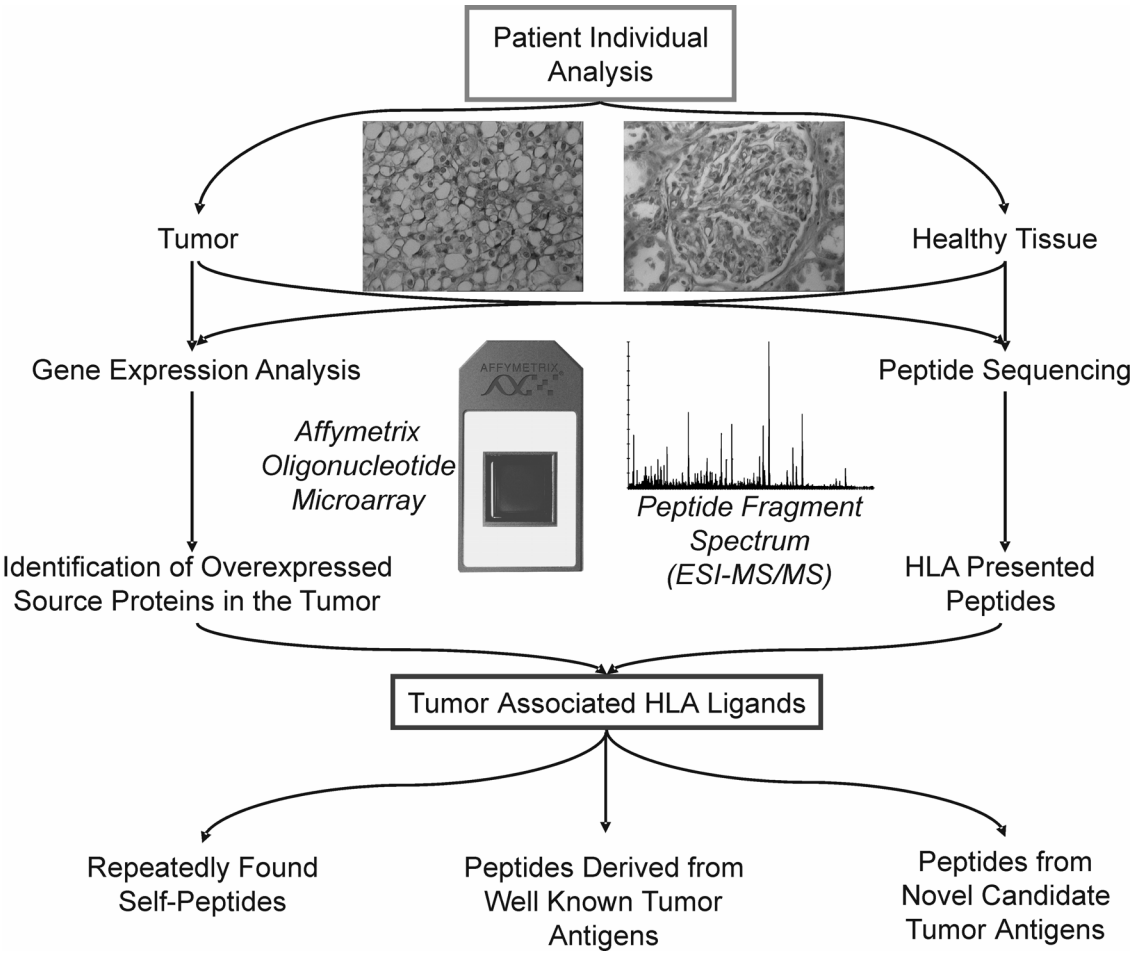

Figure 2.1.1.1. Patient individual analysis of gene expression patterns and HLA-presented peptides.

During our investigations we were able to sequence peptides from classical TAAs such as carbonic anhydrase 9 (CA9) and met proto-oncogene (MET), as well as from constitutively or individually upregulated proteins such as insulin-like growth factor binding protein 3 (IGFBP3), adipophilin (ADFP) and apolipoprotein L1 (APOL1). Here, we describe the results of a systematic analysis of peptide presentation patterns and gene expression profiles in 13 RCC patients.

2.1.1.3 Materials and Methods

Patients and tumor specimens

Surgically removed RCC specimens (Table 2.1.1.1) were provided by the Department of Urology, University of Tübingen, after written informed consent

had been obtained from each patient. Specimens were snap frozen in liquid nitrogen immediately after surgery. Pathological staging and grading was performed by the Department of Pathology and HLA typing was done by the Department of Transfusion Medicine, University of Tübingen. This study has been approved by the local ethical review board.

Table 2.1.1.1. Renal cell carcinoma specimens included in the study. Peptide data were generated from all samples while gene expression profiling data are available for specimens RCC44 - RCC130 only.

Specimen	Histology	Grade and Stage	HLA Typing
RCC01	Clear cell RCC	T3 Nx Mx (G2)	A*02; A*68; B*18; B*44
RCC13	Clear cell RCC	T2 Nx Mx (G2)	A*02; A*24; B*07; B*40
RCC44	Chromophilic RCC	T1 Nx Mx (G2)	A*03; A*11; B*27
RCC68	Clear cell RCC	T3 N0 Mx (G3)	A*02; A*29; B*15; B*45
RCC70	Clear cell RCC	T3 N1 M0 (G2)	A*01; A*02; B*07; B*08
RCC73	Clear cell RCC	T3 N0 (G2)	A*02; A*03; B*07; B*57
RCC75	Chromophilic RCC	T4 Nx M1 (G2-3)	A*03; B*07; B*40
RCC98	Clear cell RCC	T3 Nx M1 (G2-3)	A*01; A*03; B*07; B*18
RCC103	Clear cell RCC	T3 N0 Mx (G2)	A*11; A*25; B*15; B*44
RCC112	Metastasis of clear cell RCC in the adrenal gland		A*01; A*31; B*08; B*27
RCC115	Clear cell RCC	T3 N0 Mx (G2)	A*02; A*03; B*15; B*18
RCC116	Clear cell RCC	T3 N2 Mx (G2)	A*01; A*02; B*27; B*37
RCC130	Clear cell RCC	T1 N1 Mx (G3)	A*02; A*24; B*07; B*44

Peptide isolation and sequencing

Frozen tumor tissue was processed as described previously [4]. Peptides were isolated according to standard protocols [5] using the HLA class I specific antibody W6/32.

For RCC01 and RCC44-75, eluted peptide mixtures were separated offline by reversed-phase high-performance liquid chromatography (SMART system, µRPC C2/C18 SC 2.1/10; Amersham Pharmacia Biotech, Freiburg, Germany) and fractions were analyzed by nano-ESI MS on a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF; Micromass, Manchester, UK) as described previously [4]. For RCC13 and RCC98-130, peptide mixtures were separated and analyzed online by a reversed phase Ultimate HPLC system (Dionex, Amsterdam, Netherlands) coupled directly to the mass spectrometer as described [6].

Fragment spectra were analyzed manually and database searches (National Center for Biotechnology Information, Expressed Sequence Tag) were carried

out using Multiple Alignment System for Protein Sequences Based on Three-way Dynamic Programming (MASCOT, http://www.matrixscience.com).

Peptide synthesis

Synthetic peptides were synthesized in an automated peptide synthesizer EPS221 (Abimed, Langenfeld, Germany) following the 9-fluorenylmethyl-oxycarbonyl/tert-butyl (Fmoc/tBu) strategy as described [4].

Gene expression analysis by high-density oligonucleotide microarrays

Frozen fragments of tumors RCC44-130 were homogenized by mortar and pestle under liquid nitrogen. Total RNA was prepared from these samples using TRIzol (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol, followed by a cleanup with RNeasy (QIAGEN, Hilden, Germany). Total RNA from healthy human tissues was obtained commercially (Ambion, Huntingdon, UK; Clontech, Heidelberg, Germany; Stratagene, Amsterdam, Netherlands). The RNA from several individuals (between 2 and 62 individuals) was mixed in a way that RNA from each individual was equally weighted. Quality and quantity were assessed on an Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany) using the RNA 6000 Pico LabChip Kit (Agilent).

Gene expression analysis of all RNA samples except RCC130 was performed by Affymetrix Human Genome U133A oligonucleotide microarrays (Affymetrix, Santa Clara, CA). For RCC130, HG-U133 Plus 2.0 was used. The same normal kidney sample was hybridized to both array types to achieve comparability. All steps were carried out according to the Affymetrix manual (www.affymetrix.com/support/technical/manual/expression_manual.affx).

Briefly, double-stranded cDNA was synthesized from 5-8 µg of total RNA using SuperScript RTII (Invitrogen) and the oligo-dT-T7 primer (MWG Biotech, Ebersberg, Germany) as described in the manual. *In vitro* transcription was performed with the BioArray™ High Yield™ RNA Transcript Labeling Kit (ENZO Diagnostics, Inc., Farmingdale, NY) for the U133A arrays or with the GeneChip IVT Labeling Kit (Affymetrix) for the U133 Plus 2.0 arrays, followed by cRNA fragmentation, hybridization, and staining with streptavidin-phycoerythrin and biotinylated anti-streptavidin antibody (Molecular Probes, Leiden, Netherlands). Images were scanned with the Agilent 2500A GeneArray Scanner (U133A) or the Affymetrix GeneChip Scanner 3000 (U133 Plus 2.0) and data were

analyzed with the MAS 5.0 (U133A) or GCOS (U133 Plus 2.0) software (Affymetrix) using default settings for all parameters. Pairwise comparisons were calculated using the respective normal kidney array as baseline. For normalization, 100 housekeeping genes provided by Affymetrix were used (http://www.affymetrix.com/support/technical/mask_files.affx). Relative expression values were calculated from the signal log ratios given by the software and the normal kidney sample was arbitrarily set as 1.

2.1.1.4 Results and discussion

Patient-individual analysis of tumor-associated peptides presented on RCC.

LC/MS-based peptide sequencing of HLA ligands extracted from surgically removed RCC specimens yielded approximately 100 different peptides per patient. However, a lot more peptides are expected to be presented by tumor cells, so we estimate that we still detect only the most abundantly presented peptides, which make up just a few percent of the whole HLA "ligandome" [6]. From 13 primary RCC samples we were able to sequence more than 700 different peptides using fragmentation-induced mass spectrometry (supplementary Table S1, http://www.uni-tuebingen.de/uni/kxi/PaperSupplements/CII_S1.pdf). These peptides derived from more than 500 different source proteins and were presented by various HLA allotypes. The following assignments of peptide sequences to specific allotypes are solely based upon known binding motifs in connection with the HLA typing of the samples and not on direct experimental evidence. All natural HLA ligands will be included in the next update of the HLA ligand database SYFPEITHI (www.syfpeithi.de). With regard to their expression profiles, the source proteins of HLA ligands could be divided into three groups. Firstly (i), as expected, only a small percentage of the peptides identified were of relevance with regard to tumor immunotherapy. Most peptides derived from structure proteins, constitutively expressed enzymes, and receptors, and represented classical self-peptides. More important (ii), we were able to define various peptides derived from well-known tumor-associated antigens such as carbonic anhydrase 9 (CA9), met proto-oncogene (MET), and adipophilin (ADFP) which have already been used for vaccination in several patients in an ongoing clinical trial. Additionally (iii) we identified several novel antigens such as apolipoprotein L1 (APOL1), matrix metalloproteinase 7

(MMP7), insulin-like growth factor binding protein 3 (IGFBP3), regulator of G-protein signalling 5 (RGS5) and acyl-CoA synthetase long-chain family member 4 (ACSL4).

In general, we considered an antigen overexpressed if the mRNA expression of its source protein was increased at least three-fold in the respective tumor compared to normal kidney and also markedly increased compared to other healthy tissues. With the knowledge of HLA ligands derived from such overexpressed antigens, vaccination cocktails can be designed which aim at individually distinct characteristics of the patient´s malignancy.

Constitutively expressed structure proteins are a major source of HLA class I presented peptides.

The majority of peptides which were sequenced throughout our analysis derived from housekeeping proteins such as vimentin, actin, or spectrin. For instance, we were able to sequence 14 different peptides from vimentin restricted to several different HLA subtypes (Table 2.1.1.2). So far our peptides cover nearly 26% of the 466 amino acid sequence of vimentin; they were found on 8 of 13 investigated tumors. From no other source protein were more peptides defined, underlining the observation that clear cell renal cell carcinomas express vimentin to a high extent [7]. A median of 3.5-fold overexpression of vimentin in comparison to normal kidney tissue (range 0.2-6.4) was determined, but only a 1.9-fold overexpression if compared to the median of all other healthy tissues (median 1.6; range 0.3-6.1). The ubiquitous expression of vimentin and the resulting widespread presentation of vimentin-derived peptides on healthy tissues exclude vimentin peptides from usage for vaccination. The same is true for other structure proteins: six different peptides were found from β-actin, five from non-erythrocytic beta, and three from alpha spectrin. Adipophilin, a tumor-associated antigen we recently identified [8, 9], was the only exception which represented a non-structural protein.

Table 2.1.1.2. Proteins from which abundant HLA ligands were repeatedly found.

Source Protein Overexpression > 3-fold in X/11 RCCs (M, median; R, range of overexpression)	Entrez Gene ID	Peptides Found on X/13 Tumors	Sequence	HLA Restriction (n.a.: not assigned)
vimentin (VIM)	7431	8	ALRDVRQQY	B*1501
6/11 (M 3.5; R 0.2 - 6.4)			ALRPSTSRSLY	A*03
			DLERKVESL [38]	A*0201
			EEIAFLKKL [8]	B*18
			EENFAVEA	B*45
			MEENFAVEA	B*45
			NLRETNLDSLP	n.a.
			NYIDKVRFL	A*24
			REKLQEEML	B*40
			RETNLDSLP	n.a.
			SLYASSPGGVYATR	A*03
			SRISLPLPNF	B*27
			SSVPGVRLLQDSVDF	n.a.
			SSVPGVRLLQDSVDFSL	n.a.
adipose differentiation-related protein (ADFP)	123	5	IARNLTQQL	B*07
5/11 (M 2.6; R 0.1 - 5.5)			MAGDIYSVFR [8]	A*6801
			MTSALPIIQK [8]	A*6801
			SLLTSSKGQLQK	A*03
			SVASTITGV [8]	A*0201
			TSALPIIQK	A*03
			VQKPSYYVR	A*31
actin, beta (ACTB)	60	4	LRVAPEEHPVL	n.a.
0/11 (M 0.8; R 0.6 - 2.5)			MEKIWHHTF	B*18
			MQKEITAL	B*1501
			RVAPEEHPV	A*02
			RVAPEEHPVL	A*02
			RVAPEEHPVLLT	A*02
spectrin beta, non-erythrocytic 1 (SPTBN1)	6711	5	AVCEVALDY	n.a.
0/11 (M 0.8; R 0.5 - 2.1)			DEKSIITY	B*18
			DEMKVLVL [8]	B*18
			EEASLLHQF	B*44
			KPRDVSSVEL	B*07
myosin light chain alkali non-muscle isoform (MYL6)	4637	4	AEIRHVLVTL	B*40
0/11 (M 0.7; R 0.4 - 1.0)			EAFVRHIL	B*08
			LVRMVLNG	n.a.
			YEELVRMVL	B*40
catenin (cadherin-associated protein), alpha 1 (CTNNA1)	1495	3	FIDASRLVY	A*01
0/11 (M 0.8; R 0.5 - 1.1)			LQHPDVAAY	B*1501
			NEQDLGIQY [8]	B*44 / B*18
spectrin alpha, non-erythrocytic 1 (SPTAN1)	6709	3	ADSLRLQQL	B*37
0/11 (M 0.8; R 0.6 - 1.5)			ETFDAGLQAF	A*25
			RQGFVPAAY	B*1501

Investigation of reported tumor-associated antigens in RCC

Only few tumor-associated antigens have been described to be associated with renal cell carcinoma and suggested to serve as targets in tumor immunotherapy. We specifically searched for reported HLA ligands from these antigens and analyzed their gene expression (Table 2.1.1.3). From RAGE, PRAME, members of the MAGE family, NY-ESO-1, and from shared TAAs telomerase, survivin, and MUC-1 we detected no HLA-presented peptides

whatsoever. However, from adipophilin, MET, CA9, and cyclin D1 known and novel HLA ligands were characterized. Upregulation of their genes varied considerably (Table 2.1.1.3). Only three previously described tumor-associated antigens in renal cell carcinoma played a significant role during our analyses, the met proto-oncogene [8, 10], adipophilin [8, 9], and carbonic anhydrase 9 [11] were upregulated in the majority of the tested specimens and yielded abundant HLA ligands (see below, Table 2.1.1.2, and Table 2.1.1.3). Survivin, cyclin D1, and PRAME were overexpressed in a minority of tumors, but MUC1, TERT, and RAGE did not fulfill our overexpression criteria in one single example.

Table 2.1.1.3. Expression analysis, known T cell epitopes, and novel HLA ligands of reported RCC-associated antigens.

RCC-associated Tumor Antigen Overexpression > 3-fold in X/11 RCCs (M, median; R, range of overexpression)	Entrez Gene ID	Known T Cell Epitopes	HLA Restriction	References	Peptides Found in This Study
met proto-oncogene (MET) 11/11 (M 12.3; R 4.3 - 28.3)	4233	YVDPVITSI	A*02	[10]	YVDPVITSI (A*02) [8]
carbonic anhydrase isoform IX (CA9) 7/11 (M 4.0; R 0.4 - 11.3)	768	HLSTAFARV	A*02	[26]	SPRAAEPVQL (B*07)
adipose differentiation-related protein (ADFP) 5/11 (M 2.6 ; R 0.1 - 5.5)	123	SVASTITGV	A*02	[9]	See Table 2
cyclin D1 (CCND1) 4/11 (M 1.8; R 0.7 - 5.7)	595	RLTRFLSRV	A*02 (allo)	[39]	ETIPLTAEKL (A*6801) [8]
		LLGATCMFV	A*02 (allo)	[39]	
survivin (BIRC5) 3/11 (M 1.4; R 0.4 - 0.9)	332	ELTLGEFLKL	A*02	[40]	
preferentially expressed antigen in melanoma (PRAME) 2/11 (M 0.4; R 0.1 - 4.7)	23532	SLLQHLIGL	A*02	[41]	
		ALYVDSLFFL	A*02	[41]	
		VLDGLDVLL	A*02	[41]	
		SLYSFPEPEA	A*02	[41]	
		LYVDSLFFL	A*24	[42]	
melanoma antigen, family A, 3 (MAGEA3) 1/11 (M 1.3; R 0.4 - 6.2)	4102	FLWGPRALV	A*02	[43, 44]	
		KVAELVHFL	A*0201	[45]	
		EVDPIGHLY	A*01, B*35	[46, 47]	
		IMPKAGLLI	A*24	[48]	
		TFPDLESEF	A*2402	[49]	
		MEVDPIGHLY	B*44	[50]	
renal tumor antigen (RAGE) 0/11 (M 0.6; R 0.4 - 1.3)	5891	PASKKTDPQK	B*08	[38]	
		SPSSNRIRNT	B*07	[51]	
cancer/testis antigen 1B (NY-ESO-1) 0/11 (M 0.7; R 0.5 - 2.6)	1485	SLLMWITQC	A*0201	[52]	
melanoma antigen, family A, 1 (MAGEA1) 0/11 (M 0.1; R 0.0 - 0.6)	4100	KVLEYVIKV and many others	A*0201	[53]	
mucin 1 (MUC1) 0/11 (M 0.4; R 0.1 - 0.8)	4582	LLLLTVLTV	A*02	[54]	
		STAPPVHNV and others	A*0201	[54, 55]	
telomerase reverse transcriptase (TERT) 0/11 (M 0.6; R 0.5 - 1.5)	7015	ILAKFLHWL	A*0201	[56]	
		KLFGVLRLK	A*03	[57]	
		VYGFVRACL	A*2402	[58]	
		VYAETKHFL	A*2402	[58]	

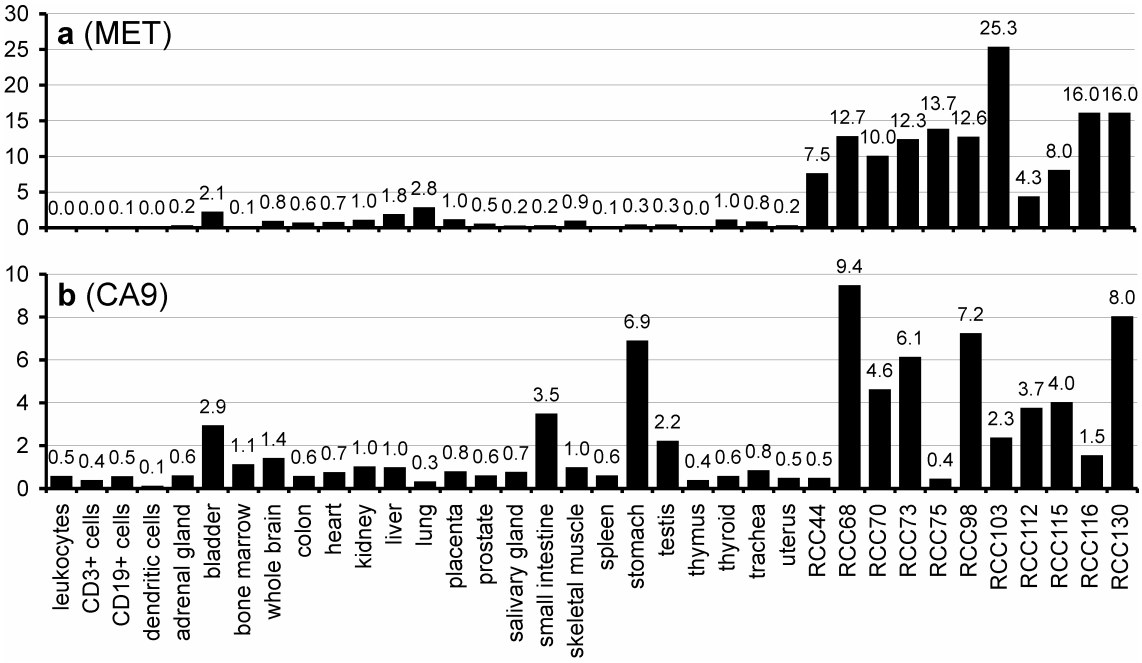

Figure 2.1.1.2. mRNA expression profiles of met proto-oncogene (MET) (a) and carbonic anhydrase IX (CA9) (b) in 11 analyzed RCCs and various human tissues. Relative expression values are normalized to kidney (expression = 1). MET appeared highly overexpressed in all tumors, CA9 was overexpressed over kidney in most tumors, while stomach and small intestine also show high expression of CA9.

Both, met proto-oncogene [12] and carbonic anhydrase 9 [11] are known to be expressed by the vast majority of renal cell carcinomas. This was confirmed by our data: MET mRNA was upregulated in all analyzed RCCs by 12.3-fold in average in comparison to healthy kidney tissue (Table 2.1.1.3) with no relevant expression in all other human tissues (Figure 2.1.1.2a). The HLA-A*02-presented CTL epitope YVDPVITSI [8] was previously shown to mediate tumor cell lysis *in vitro* [10]. Carbonic anhydrase 9 (CA9; G250), the only known tumor-associated isoform of carbonic anhydrase [11, 13], is also expressed by a set of other malignancies, for example breast cancer [14], non-small-cell lung cancer [15], and squamous cell head and neck cancer [16, 17]. CA9 expression in general is hypoxia-inducible, and was suggested to be an endogenous marker for tumor hypoxia [15]. The frequent deletion of the von Hippel-Lindau tumor suppressor gene (VHL) in the case of renal cell carcinoma is associated with the upregulation of CA9, a characteristic antigen for RCC [18]. The level of CA9 expression was even shown to be an independent prognostic marker for this disease [19]. *In vivo* studies show that the monoclonal anti-CA9 antibody G250 exclusively binds to tumor cells, and that CA9 can be used as a therapeutic target [20-22]. In consequence, it has been targeted in various investigational therapeutic approaches in renal cell carcinoma [23-25] and is

also considered a suitable source of epitopes in CTL-based immunotherapy. Although we could not detect the prominent HLA-A*02-restricted CTL epitope, HLSTAFARV [26], the HLA-B*07-presented CA9 peptide SPRAAEPVQL was sequenced by collision-induced tandem mass spectrometry (Figure 2.1.1.3) and represents a promising candidate for peptide-based immunotherapy. CA9 was overexpressed in 7/11 RCCs as expected [27], expression in normal tissue was relevant only in stomach, small intestine, and bladder (Figure 2.1.1.2b), consistent with previous reports [21, 27].

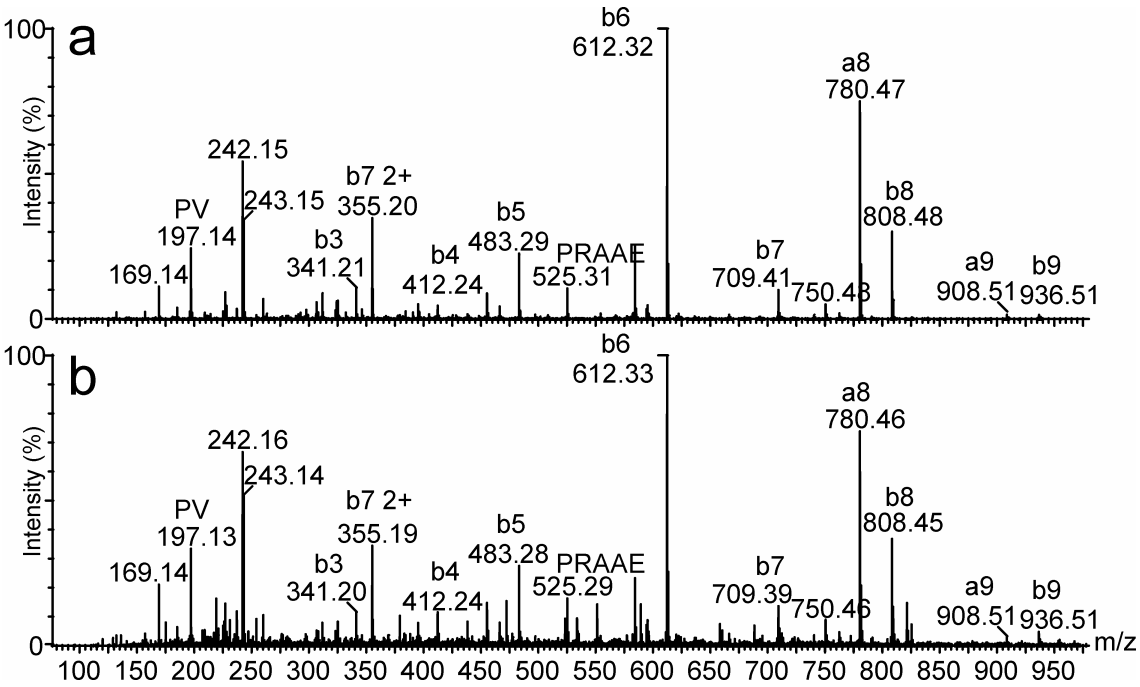

Figure 2.1.1.3. Fragmentation-induced mass spectra of the HLA-B*07-presented CA9 peptide SPRAAEPVQL. a) synthetic peptide, b) peptide extracted after immunoprecipitation of tumor HLA.

The most abundant source of HLA ligands in the group of the reported tumor antigens was adipose differentiation-related protein adipophilin (ADFP), from which peptides were detected in five of thirteen investigated tumors. This led to the characterization of seven different peptides with different HLA restrictions (Table 2.1.1.2). According to this, adipophilin ranked second among our frequent source protein for peptides after vimentin, suggesting a high abundance of adipophilin peptides on the surface of RCC cells. Adipophilin was also highly overexpressed in most renal cell carcinomas of the clear cell subtype (data not shown), whereas both chromophilic RCCs, RCC44 and RCC75, showed no upregulation of adipophilin at the mRNA level. The only tissues with relevant adipophilin expression are female mammary gland and

placenta. This suggests that adipophilin-derived peptides can be used for vaccination in male patients with renal cell carcinoma, especially in clear cell type malignancies. One of the adipophilin-derived HLA ligands, the peptide SVASTITGV presented by HLA-A*02, was recently shown to be a T cell epitope which mediates tumor cell lysis *in vitro* [9]. The novel adipophilin peptides cover a broad range of HLA restrictions (Table 2.1.1.2), and thus represent candidate vaccination peptides in our concept of patient-individual immunotherapy [28].

Complementary analysis of gene expression and peptide presentation leads to the identification of new broadly expressed tumor-associated HLA ligands.

A set of proteins was repeatedly found to be upregulated at the mRNA level and a source of HLA ligands in renal cell carcinoma (Table 2.1.1.4). These proteins are, according to their expression profiles, potential sources for vaccination peptides either in all or most patients as for insulin-like growth factor binding protein 3 (IGFBP3), apolipoprotein L 1 (APOL1), and the regulator of G-protein signalling 5 (RGS5), or only in few patients as for matrilysin (MMP7) or the acyl-CoA synthetase long-chain family member 4 (ACSL4).

Table 2.1.1.4. Novel RCC-associated antigens identified by overexpression and source of HLA ligands presented by several allotypes.

Source Protein Overexpression > 3-fold in X/11 RCCs (M, median; R, range of overexpression)	Entrez Gene ID	Sequence	HLA Restriction	References
apolipoprotein L, 1 (APOL1) 9/11 (M 7.1; R 1.1 - 40.2)	8542	FLGENISNFL ALADGVQKV	A*0201 A*0201	[8, 38] [8, 38]
insulin-like growth factor binding protein 3 (IGFBP3) 8/11 (M 6.0; R 2.0 - 10.2)	3486	RPTLWAAAL	B*07	
regulator of G-protein signalling 5 (RGS5) 7/11 (M 7.2; R 0.3 - 14.9)	8490	GLASFKSFLK LAALPHSCL	A*03 A*02	
matrix metalloproteinase 7, matrilysin (MMP7) 4/11 (M 2.4; R 0.3 - 12.1)	4316	FPNSPKWTSK SLFPNSPKWTSK	A*03 A*03	
cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1) 3/11 (M 0.6; R 0.3 - 9.2)	1545	FLDPRPLTV	A*02	
acyl-CoA synthetase long-chain family member 4 (ACSL4) 1/11 (M 1.3; R 0.8 - 7.1)	2182	KLFDHAVSKF VPNQKRLTLL	A*03 B*07	

Insulin-like growth factor 1 (IGF1) was shown to be involved in the progression of malignancies derived from proximal tubule epithelial cells, and insulin-like growth factor binding proteins, among them IGFBP3, are known to be

upregulated in clear cell renal cell carcinoma [12, 29, 30]. In our investigations, IGFBP3 was upregulated in at least 8 of 11 analyzed specimens (Table 2.1.1.4) with no relevant expression in normal tissues. One *in vivo* processed peptide from IGFBP3, RPTLWAAAL, was presented by HLA-B*07.

The expression profile of APOL1 also suggests tumor association: Nine of eleven analyzed clear cell carcinomas showed an upregulation of APOL1 in comparison to normal kidney (Table 2.1.1.4), although the factors of overexpression were rather heterogeneous (Figure 2.1.1.4a). The repeated detection of HLA ligands derived from APOL1 and its extensive overexpression in tumors RCC68, RCC98, RCC115, and RCC130 justifies the usage of APOL1 peptides for vaccination in these patients according to our criteria.

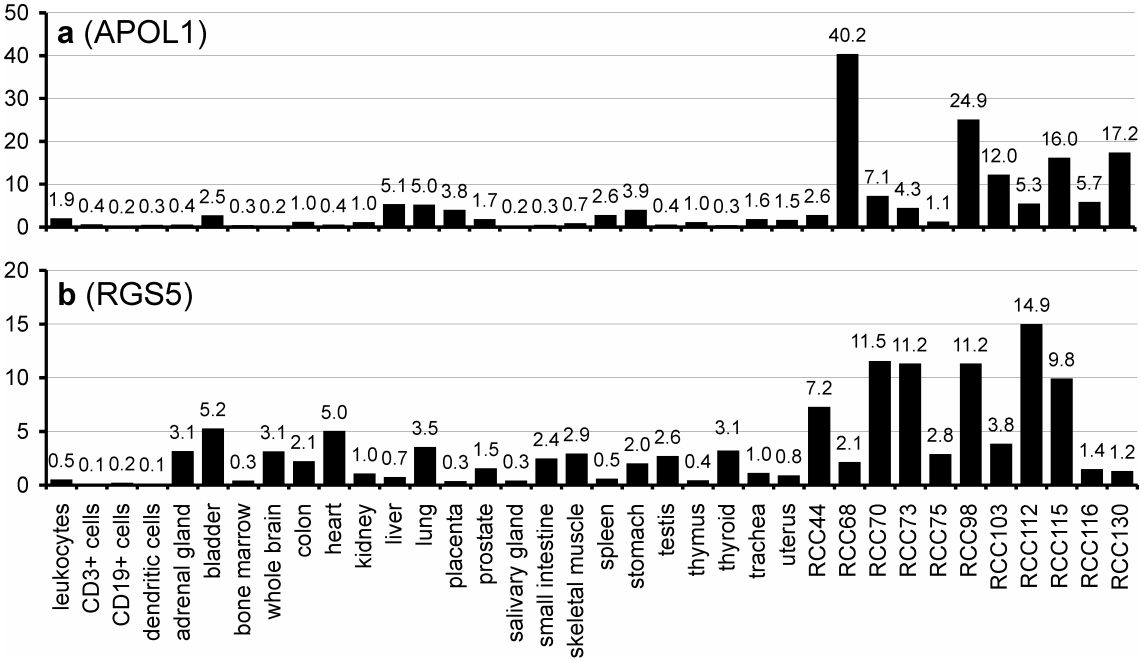

Figure 2.1.1.4. mRNA expression profiles of a) apolipoprotein L 1 (APOL1) and b) regulator of G-protein signalling 5 (RGS5). APOL1 is extensively upregulated in RCC68, RCC98, RCC115, and RCC130. RGS5 appears overexpressed in RCC70, RCC73, RCC98, RCC112, and RCC115.

RGS5, from which two HLA ligands were detected, was upregulated in seven of eleven tested tumors (Table 2.1.1.4) and reported to be overexpressed in RCCs previously [12, 31]. However, in comparison to the other healthy tissues, RGS5 shows a very heterogeneous pattern of expression (Figure 2.1.1.4b), which necessitates an individual expression analysis of each tumor before its peptides are used for vaccination.

Some HLA ligands are tumor-associated candidates in individual cases only.

MET, CA9, ADFP, IGFBP3, and APOL1 represent antigens overexpressed in all or most renal cell carcinomas. Such antigens provide a source for vaccination peptides per se, even if only few naturally processed peptides are known. However, individual patterns of gene expression may be found in individual cancer specimens. Therefore, we place our emphasis on a patient-individual concept of immunotherapy and perform individualized gene expression and peptide analysis [28]. In this individual approach we also use peptides derived from genes that are exclusively upregulated in one or only few patients.

One example of such an antigen is matrix metalloproteinase 7 (MMP7), which was shown to be expressed in cancer cells of various origins and to play a role in the process of metastasis [32-34]. Apart from high expression levels in RCCs 68, 98, and 116 (Table 2.1.1.4), we detected relevant MMP7 expression only in the bladder. The HLA-A*03 ligand SLFPNSPKWTSK, as well as its shorter variant FPNSPKWTSK, were found on RCC75 and RCC98. It has to be mentioned that for these peptides as well as for all other HLA ligands described in this and the preceding chapter no data on T-cell reactions exist so far.

The acyl-CoA synthetase long-chain family member 4 (ACSL4) was overexpressed in one patient of the chromophilic subtype, RCC75 (Table 2.1.1.4). ACSL4 overexpression was recently reported to be associated with colon adenocarcinoma [35] and hepatocellular carcinoma [36]. From RCC75, the HLA-A*03-presented peptide KLFDHAVSKF was characterized and later also found in RCC98. ACSL4 stands for an antigen which might be used for vaccination only in particular cases.

Gene expression profiles from 11 RCCs allow for the identification of novel candidate RCC antigens.

While gene expression analysis yields comprehensive data, HLA ligand characterization does not: From the estimated over 10,000 peptides making up the HLA class I ligandome of a given cell, only a very low percentage can be identified with current tools and strategies. In contrast, almost every gene of the human genome can be assessed by gene expression profiling.

Therefore, we searched our gene expression data for genes upregulated in most tumors in relation to healthy tissues, even without identified HLA ligands.

Here we present three promising antigens that emerged from these analyses. The tumor necrosis factor (ligand) superfamily, member 7 (TNFSF7), was overexpressed in 5 of 11 tumors (Figure 2.1.1.5a). Ubiquitin D (UBD) is characterized by undetectable expression in most healthy tissues but strong expression in clear cell RCCs (Figure 2.1.1.5b). One peptide from UBD, DANPYDSVKKI (HLA-B*51) has recently been identified [37]. Our third example of a consistently overexpressed protein in RCC (nine of eleven tested tumors with a more than 18-fold overexpression) is the regulator of G-protein signalling 1, RGS1 (Figure 2.1.1.5c). In contrast to RGS5, which has already been described as upregulated in RCC [12, 31], RGS1 has not yet been mentioned in the context of RCC. Interestingly, no other members of the RGS family were overexpressed in our RCC samples. Unfortunately, no HLA-presented peptides from RGS1 are known so far.

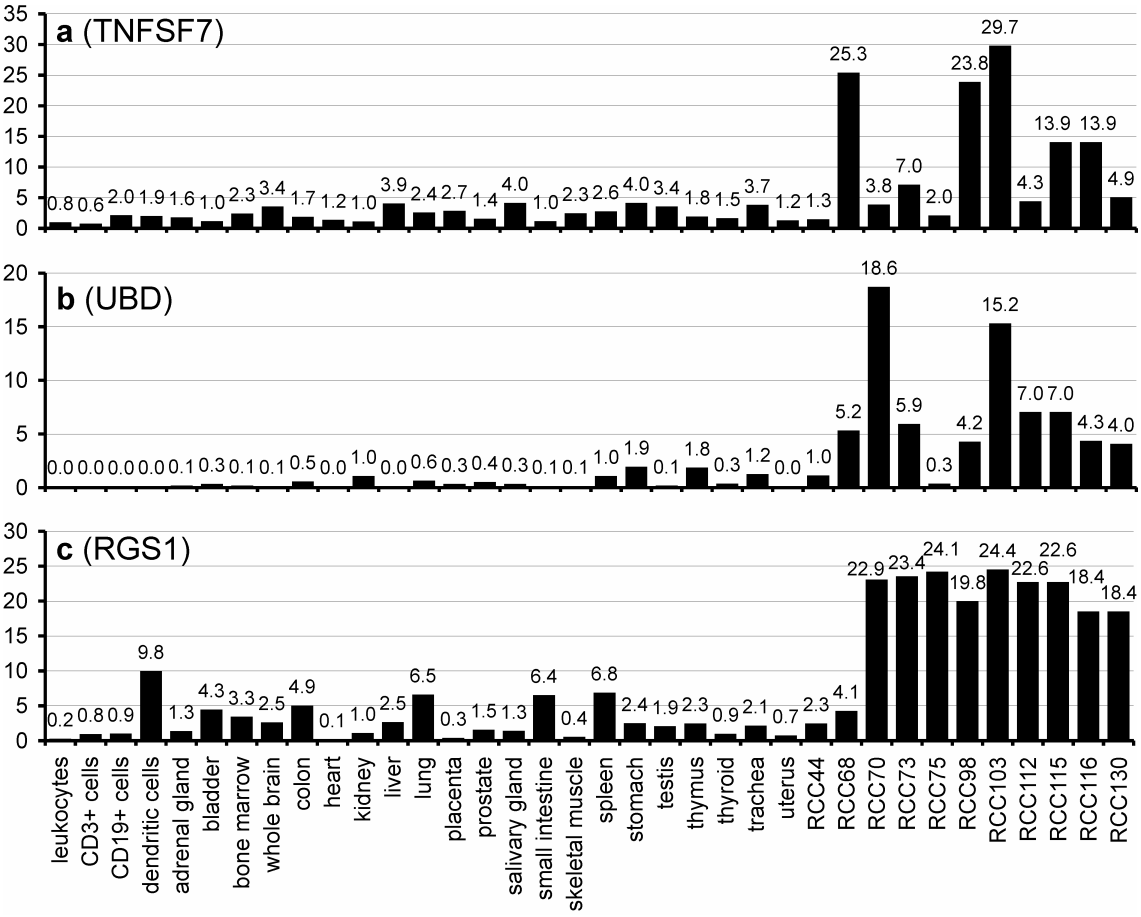

Figure 2.1.1.5. Novel potential RCC-associated antigens, identified by their overexpression in RCC. a) Tumor necrosis factor (ligand) superfamily, member 7 (TNFSF7), b) Ubiquitin D (UBD), c) Regulator of G- protein signalling 1 (RGS1). HLA-presented peptides have only been identified for UBD so far.

Conclusions

In this report we present data resulting from a systematic large scale analysis of HLA peptide presentation patterns and mRNA expression profiles in renal cell carcinoma. We identified a number of novel HLA ligands from reported RCC antigens such as adipophilin and CA9 and confirmed the constitutively high expression of the classical antigens CA9, ADFP and MET, whereas no evidence was revealed for a concurrent elevated expression level of most other previously suggested tumor associated antigens. Various proteins constitutively or sporadically overexpressed in RCC were suggested tumor-associated antigens, for example RGS5, RGS1, IGFBP3, and APOL1. From some of these proteins novel HLA class I peptides were characterized that might turn out to represent target epitopes for CTL responses. Future T cell work will have to reveal the immunogenicity of these peptides. The therapeutic impact of a vaccination treatment with the mentioned peptides is currently under intensive investigation.

Acknowledgements

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2.1.2 Metastases show an expression profile similar to the primary tumor

2.1.2.1 Introduction

Radical nephrectomy is the standard treatment for renal cell carcinoma (RCC) [1]. In consequence, after removal of the primary tumor any kind of follow-up therapy must aim at residual tumor cells or manifest metastases. In most cases, comprehensive analyses are only possible for the resected primary tumor. This especially applies to HLA ligand analyses described in chapter 2.1.1, since rather large amounts of material are necessary for this approach. Against this background it is important to ask whether residual tumor cells display the same characteristics as the primary tumor, which is generally used for therapeutic target selection but is not the actual target during therapy.

The prevailing model of cancer progression assumes that primary tumors consist of heterogeneous subpopulations of tumor cells, which have different biological characteristics and are the product of neoplastic progression - an evolutionary process involving multiple stages and leading to several distinct tumor cell populations [2]. It is further postulated that most primary tumor cells have low metastatic potential and only very rare cells within those tumors acquire metastatic capacity through somatic mutation [3]. This implies that heterogeneous bulk primary tumor masses might be very different from metastases, which have their clonal origin in those rare cells. This model, however, has been recently challenged by a hypothesis suggesting that the tendency to metastasize is largely determined by mutant alleles acquired early during multistep tumorigenesis [4]. This alternative model has been heavily debated [5-7] but would be very attractive if it proved to be true in general, because - among other advantages - target selection for therapy based on primary tumors could be more easily justified and would more likely be successful.

Various microarray gene expression studies on primary tumors have been performed in order to predict their metastatic potential and select appropriate treatment strategies [8-10]. However, only few direct comparisons of primary tumors and metastases have been published [11-15]. For renal cell carcinoma, only one such experiment has been described [16] using cultured cell lines originating from either primary tumors or metastases. This approach, however,

takes the tumor cells out of their natural microenvironment and therefore bears the risk of considerably altered expression patterns [17].
The following experiment addresses the gene expression of a primary renal cell carcinoma specimen, autologous normal kidney tissue, and two hilar lymph node metastases.

2.1.2.2 Materials and Methods

From the patient RCC190, surgically removed material from the primary tumor (RCC190T), normal kidney (RCC190N), and two hilar lymph node metastases (RCC190M1 and RCC190M2) was obtained. RNA was isolated and gene expression profiling was performed using Affymetrix HG-U133Plus 2.0 microarrays containing approx. 54,000 probesets as described in chapter 2.1.1.3. Commercially obtained normal kidney RNA (Clontech, Heidelberg, Germany) was also hybridized to the same array type and used as reference for comparison of differential expression.

2.1.2.3 Results and Discussion

Gene expression analysis by Affymetrix arrays provides several different parameters which can be used to assess the similarity of expression profiles. Figure 2.1.2.1A shows genes detected as present in the different samples of RCC190. The absolute numbers were quite similar for all samples. The overlap between tumor and metastases was slightly higher than between normal and malignant (tumor, metastasis 1, metastasis 2) samples. However, the rather small differences observed in this parameter do not allow any further interpretation. The picture becomes clearer if quantitative expression differences are taken into consideration. In order to assess those, RCC190 samples were compared to pooled normal kidney RNA as a neutral reference. Figure 2.1.2.1B displays the number of genes which were at least two-fold overexpressed against this reference. Interestingly, the RCC190 normal kidney already showed a considerable number of overexpressed genes against the reference kidney but not as many as the malignant samples. The overlap of overexpressed genes identified in common between normal and malignant samples was about 50%, whereas the overlap among the three malignant samples was around 80%. This indicates that the expression signature of the

metastases is much more closely related to the primary tumor than to the autologous normal kidney and is in the same range as the between the metastases themselves. Altogether, these results suggest that differences between normal and malignant expression signatures for RCC are not so much characterized by a larger number of genes whose transcription is entirely turned on or off during tumorigenesis. In fact, altered profiles rather become manifest in quantitative changes observed for mRNA species characterizing the specific state of the sample.

A	Normal	Tumor	Metastasis 1	Metastasis 2
Normal	19939	83%	82%	85%
Tumor	15003	18195	86%	90%
Metastasis 1	15636	15602	18975	91%
Metastasis 2	16915	16431	17259	21737

B	Normal	Tumor	Metastasis 1	Metastasis 2
Normal	2457	41%	56%	55%
Tumor	1005	2929	82%	72%
Metastasis 1	1366	2401	3973	78%
Metastasis 2	1356	2117	2814	3626

Figure 2.1.2.1. Genes identified in common between different samples from RCC190. (A) Genes detected as "present" according to the detection call algorithm of the GCOS software. Black cells show the number of present genes for each sample. The lower left part of the table indicates the number of such genes that were found in common between the different sample combinations. The grey shaded upper right part of the tables shows the corresponding percentages. The smaller number of present genes among the two arrays constitutes the maximum number that could be detected in common and was therefore set as 100 percent. **(B) Genes upregulated at least twofold compared with normal reference kidney.** To be considered as overexpressed, genes had to fulfill general criteria for upregulation: an “increase” call together with a “present” call on the indicated array. Furthermore, a threshold criterion of twofold increase had to be met. Black cells show the number of such genes for each sample compared with reference kidney. The lower left part of the table indicates the number of genes that were found in common between the different sample combinations. The grey shaded upper right part of the tables shows the corresponding percentages. The smaller number of upregulated genes among the two array pairs constitutes the maximum number that could be detected in common and was therefore set as 100 percent.

The most comprehensive approach of assessing quantitative expression differences consists in not only looking at the numbers of genes above a certain threshold but on the relative overexpression of every gene itself. This method is illustrated in Figure 2.1.2.2.

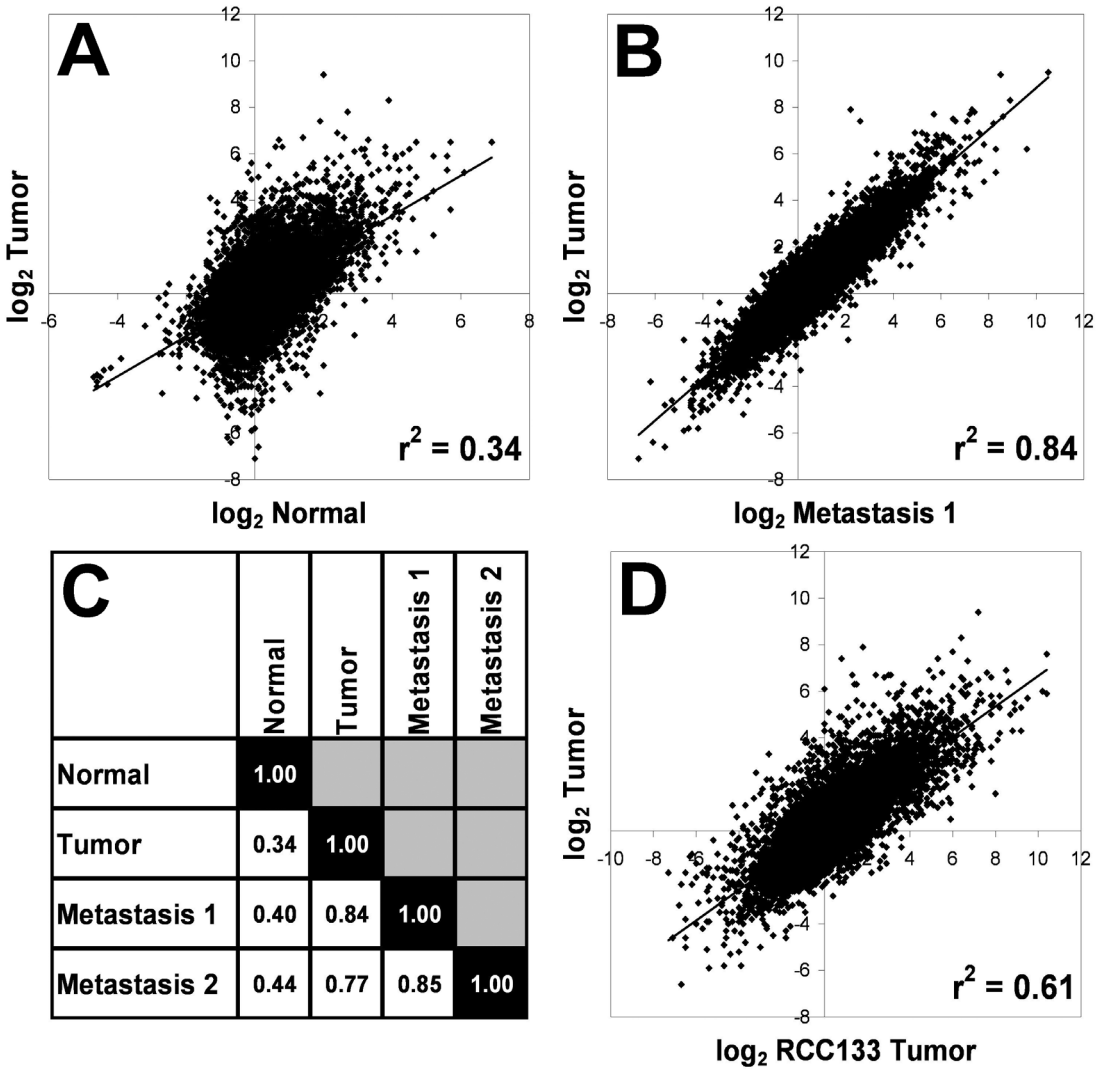

Figure 2.1.2.2. Correlation of differential expression measured for different samples against normal reference kidney. Expression differences between RCC190 samples and reference kidney were quantitatively determined by baseline comparisons using the GCOS software. The $\log_2$ values for differential expression obtained for each array pair (indicated RCC190 sample versus reference kidney) were compared with the other pairs: **(A)** Comparison of RCC190 tumor (versus reference kidney) with autologous RCC190 normal kidney (versus reference kidney). **(B)** RCC190 tumor compared with RCC190 metastasis 1. **(C)** Correlation coefficients r^2 for all comparisons between sample pairs from RCC190. **(D)** Comparison of RCC190 tumor with another representative renal cell carcinoma primary tumor (RCC133). The average correlation coefficient of comparisons between 10 different RCC tumors was $r^2 = 0.60$. Theses analyses were restricted to genes that were detected as present on both microarrays of each comparison. This pre-selection of genes was necessary in order to avoid measuring differential expression against background levels which would result in meaningless numbers inappropriate for measuring correlation in this way.

The maximum correlation is observed between the two metastases ($r^2 = 0.85$, Figure 2.1.2.2C), shortly followed by the comparison of the metastases with the primary tumor ($r^2 = 0.84$ and 0.77, Figure 2.1.2.2B and C). All correlations between the autologous normal kidney and the malignant samples are much weaker ($r^2 = 0.34$ to 0.44, Figure 2.1.2.2A and C). Additionally, correlation coefficients were calculated for RCC190 tumor compared with nine further

primary RCC tumors from other patients using the same method. The average correlation was r^2 = 0.60 (data not shown). One representative example is shown in Figure 2.1.2.2D. These results indicate that the transcriptional profile between a primary tumor and its autologous metastases is more closely related than the profile between different primary tumors and that all malignant profiles are very different from autologous normal kidney.

In order to obtain a well-substantiated picture of how closely related the malignant samples actually are, it is necessary to know the technical deviation from the ideal correlation of r^2 = 1 inherent to the method. A sound estimation of this level of noise can be achieved by replicate experiments like those performed for another part of this thesis (chapter 2.2.2). Figure 2.2.2.4D shows that - starting always from the same RNA sample - for two microarray pairs processed according to the same protocol, the correlation of differential expression is r^2 = 0.92. If these samples are compared with an array processed according to a slightly different protocol but still using the same starting RNA, the correlation decreases to r^2 = 0.89 and 0.88, respectively.

Thus, the observed correlations among the malignant samples are close to the level which corresponds to inevitable technical noise and therefore suggest that the expression signatures between the primary tumor and its metastases might in fact be identical. This finding is very positive for all therapeutic approaches relying on the analysis of primary tumors in order to find targets also applicable to fight metastases. It can be expected that if the expression profiles between tumor and metastases are so similar, this will apply to all relevant characteristics as well, even though this assumption cannot be easily verified.

These results are supported by some of the initially mentioned microarray studies of other tumor species and metastases. For breast cancer, it has been reported that metastasis and primary tumor were as similar in their expression pattern as were repeated samplings of the same primary tumor and more similar to each other than either was to any other tumor sample from different patients [11]. On the other hand, genes differentially expressed in metastasis compared with primary tumors can also be identified [14]. Arguably the most important application for microarrays in cancer research is diagnostic marker identification. Various publications demonstrate that it is possible to identify gene expression signatures in primary tumors that predict their metastatic potential or even the clinical outcome of the disease [8-10, 18]. The success of

such approaches indicates that the tendency to metastasize is very likely inherent to the majority of cells within the primary tumor. These results are strong evidence against the conventional model [3] claiming that metastases arise from rare special cells within primary tumors. If this were true, it would be impossible to recognize metastatic potential from overall tumor profiles. In contrast, all available data are in favor of the alternative hypothesis suggesting that the tendency to metastasize is acquired early during tumorigenesis [4]. Moreover, this model is strongly supported by a recent comprehensive microarray study of primary tumors and metastases [13]. Based on similar expression signatures in metastases compared with a subset of different primary tumors, the authors claim that their data "support a model in which the propensity to metastasize reflects the predominant genetic state of a primary tumor rather than the emergence of rare cells with the metastatic phenotype".
Altogether, the similarities between a primary RCC tumor and two hilar lymph node metastases demonstrated in this experiment together with ample evidence from the literature strongly suggest that tumors and their metastases might be so closely related, that target identification on primary tumors in order to fight metastasis can be justified.

2.1.2.4 References

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2.2 Potential tumor antigens in colorectal carcinoma

2.2.1 Gene expression analysis after laser microdissection

2.2.1.1 Introduction

Colorectal carcinoma as well as healthy colon tissue displays a very heterogeneous morphology if compared for example with kidney tissue (Figure 2.2.1.1). Thus, RNA isolated from crude tissue samples constitutes a mixture representing an overlay of various cell types. It may be argued that examining the expression profile of the complete cellular microenvironment of a solid tumor provides the best overall perspective of the disease process. Nevertheless, such an approach holds the risk of losing essential information by diluting pronounced changes of specific genes in a certain cell type to insignificant levels due to the possibility that there is no change in expression in the majority of cells.

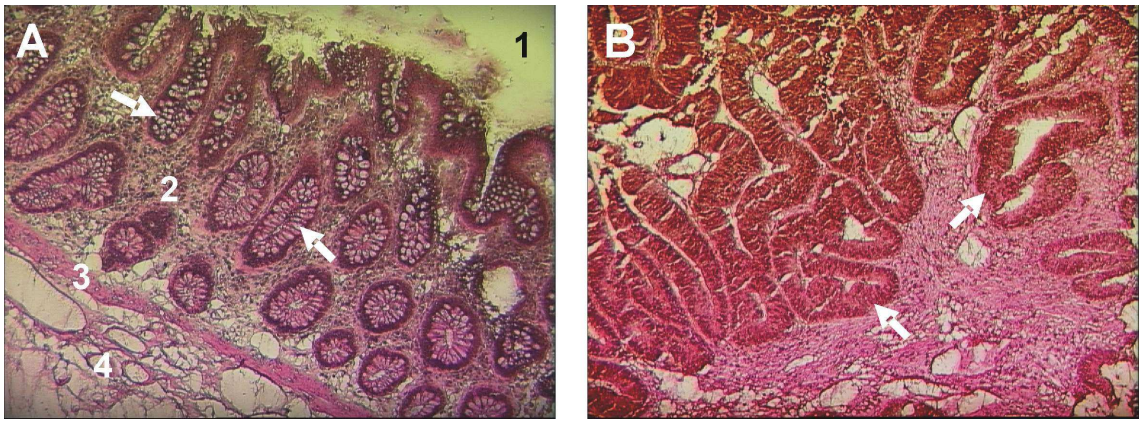

Figure 2.2.1.1. HE-stained sections of human colon specimens. **(A)** Normal colonic mucosa (area 2) is characterized by crypts (arrows) which are open to the luminal side (area 1) and are lined by epithelial goblet cells. The mucosa is separated from the submucosa (area 4) by the lamina muscularis mucosa (area 3). **(B)** Colon adenocarcinoma arises from epithelial goblet cells. Tumor cells are frequently still arranged in crypt-like structures (arrows) surrounded by non-transformed connective tissue.

Colon adenocarcinomas originate from epithelial goblet cells that are located in the colonic mucosa (Figure 2.2.1.1) [1], whereas surrounding cell types remain largely unaffected. Therefore, in order to identify tumor-associated antigens (TAA) in colon carcinoma, it is reasonable to specifically isolate tumor cells and their normal epithelial precursors and perform differential analyses on such homogeneous cell populations.

Among the most precise and efficient techniques for the isolation of specific cells from solid tissues are laser-assisted methods, which have been used for

around 30 years [2]. With the invention of laser capture microdissection (LCM) in 1996 [3], a larger number of scientists got access to such tools. Since then, a variety of platforms using similar principles have been introduced. For the studies described here, laser microbeam microdissection (LMM) followed by laser pressure catapulting (LPC) was employed [4].

The amount of material which can be acquired by laser microdissection with reasonable efforts does not usually yield enough RNA for a direct microarray analysis based on standard protocols. In fact, additional amplification steps are necessary to obtain sufficient amounts of labeled target for microarray hybridizations. Most frequently employed amplification protocols are based on one of two different methodical approaches: PCR or *in vitro* transcription. A very important requirement for all such approaches is the maintenance of the original transcriptional signature, especially between different samples to be compared. While PCR-based techniques [5, 6] appear to be ideal to generate sufficient target molecules from minute starting amounts, they always bear the danger of introducing pronounced bias due to complex hybridization kinetics during thermal cycling and the exponential amplification of the reaction. In contrast, *in vitro* transcription-based methods [7, 8] are characterized by a hybridization-independent isothermal and linear behavior, making them in theory less likely to be susceptible for distortions of the original profile. Therefore, methods using two sequential rounds of *in vitro* transcription are the most widely used for microarray analysis starting from small amounts of RNA.

2.2.1.2 Materials and Methods

Tissue Samples

Surgically removed CCA specimens and autologous normal colon tissue were provided by the Department of General Surgery, University of Tübingen, after written informed consent had been obtained from each patient. Specimens were snap frozen in liquid nitrogen immediately after surgery. This study has been approved by the local ethical review board.

Laser Microbeam Microdissection and Laser Pressure Catapulting (LMM/LPC)

Frozen tissue specimens were embedded in OCT medium (Tissue-Tek, Sakura Finetek, Zoesterwoude, NL) and stored at -80°C. 8 µm sections were cut with a

cryostat (CM3000, Leica Microsystems, Wetzlar, Germany), mounted on membrane slides (P.A.L.M. Microlaser Technologies, Bernried, Germany) and immediately fixed in ice-cold 70% ethanol. Hematoxylin/Eosin (HE) staining was performed by dipping the slides in hematoxylin (Mayer's formulation, Sigma-Aldrich, Steinheim, Germany) for 15 s, deionized water for 10 s, and 70% ethanol for 1 min, all at 4°C. The following steps were performed at room temperature: 100% ethanol for 1 min, 1% eosin (EosinY, Sigma-Aldrich, Steinheim, Germany), and 3-times 100% ethanol for 2 min each. Slides were air-dried and immediately used for laser microdissection.

Typically 3 mm^2 of normal colonic epithelial cells and tumor cells were selected and harvested using a Microbeam device (P.A.L.M.). Cells were collected in lysis buffer RLT (QIAGEN, Hilden, Germany) and total RNA was purified with an RNeasy kit (QIAGEN) including an on-column DNase I digest. RNA yield and integrity was determined using the RNA 6000 Pico LabChip kit on a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Microarray Analysis

20 ng of normal colon and tumor RNA were processed according to the two-round amplification protocol as described in chapter 2.2.2.3. Hybridization to HG-U133 Plus 2.0 oligonucleotide microarrays (Affymetrix, High Wycombe, UK), staining, and scanning were performed by the Microarray Facility Tübingen. Data analysis was done with the GCOS software (Affymetrix) as described (2.2.2.3). In order to compare gene expression in CCA samples to pooled RNA samples from healthy human tissues as described in chapter 2.1.1, the kidney sample was amplified by the two-round protocol as well. Pairwise comparisons were calculated using this kidney array as baseline.

2.2.1.3 Results and Discussion

Laser Microdissection and RNA quality

Approximately 3 mm^2 of tumor and autologous normal epithelial cells were harvested from four colon carcinoma specimens (CCA145, CCA149, CCA156, CCA165) by laser microbeam microdissection followed by laser pressure catapulting (Figure 2.2.1.2). Counting actual cell numbers in stained cryosections is virtually impossible. Therefore, areas of dissected material were

used for quantitation. 3 mm^2 of 8 µm sections yielded on average 30 ng of total RNA.

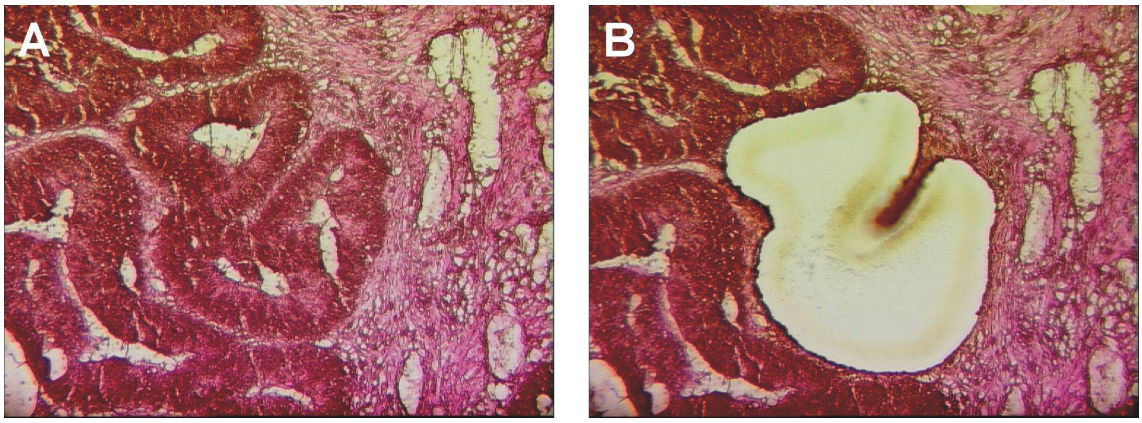

Figure 2.2.1.2. LMM/LPC of tumor cells from CCA149. (A) Before microdissection, **(B)** after dissection and catapulting. Desired cells can be precisely selected and harvested.

RNA quality constitutes an important issue in gene expression analysis of primary human samples. Especially ischemic times between surgery and further processing of the specimens are critical for RNA integrity [9, 10]. In addition, the multitude of steps necessary for further processing (i.e. cryosectioning, staining, laser dissection) bear the inherent risk of further degradation. In fact, all total RNA samples obtained from laser-dissected CCA material showed some symptoms of RNA degradation as assessed by microcapillary electrophoresis. Figure 2.2.1.3 shows representative examples of such RNA preparations. Apart from 28S and 18S ribosomal RNA (rRNA) - supposed to be present as the sole bands in intact total RNA - RNA species of intermediate and smaller sizes are visible as well, indicating degradation of rRNAs to some extent. Degradation should similarly affect mRNA and might disturb original expression profiles. However, RNA qualities comparable to those in Figure 2.2.1.3 can be routinely achieved by the described procedure and are likely to be sufficient for meaningful gene expression analysis. This is shown in the following chapter 2.2.2, where technical issues concerning gene expression analysis from small, partially degraded RNA samples are addressed in detail.

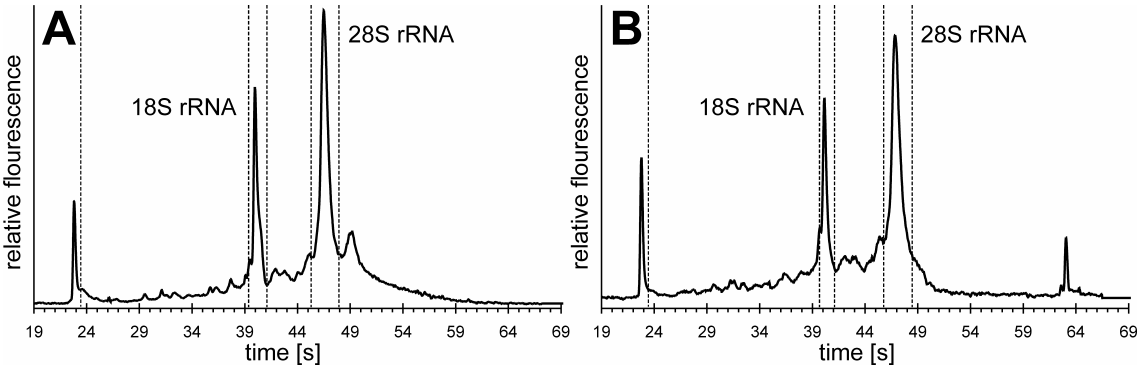

Figure 2.2.1.3. Electropherograms of RNA samples from CCA165. (A) tumor, **(B)** autologous normal cells. Analysis was performed using the RNA 6000 Pico LabChip Kit on a Bioanalyzer (Agilent). Apart from ribosomal RNA bands, degradation products are visible as well.

Identification of potential tumor associated antigens in CCA

Differential gene expression analysis was performed of CCA145, CCA149, CCA156, and CCA165 against their autologous normal epithelial cells. In addition, tumor samples were directly compared with normal kidney RNA to enable further comparisons with a larger number of healthy human tissues.

To be considered as potentially interesting tumor antigens, genes had to be strongly (at least 4-fold) upregulated in the tumor compared with autologous normal cells in two or more of the samples analyzed. The latter requirement was chosen to exclude technical outliers occurring in only one sample and in order to focus on antigens likely to be relevant not only in one single case. A further decrease of hits was achieved by requesting an "increase" (without defining a quantitative threshold) in the tumor against normal kidney. Thereby genes were filtered out which are most likely irrelevant for a potential immunotherapy due to a higher constitutive expression in another important organ. The remaining 94 genes are shown in Table 2.2.1.1. Expression profiles of these genes in comparison with a panel of healthy human tissues (see also chapter 2.1.1) were manually evaluated and genes which are promising candidates for tumor associated antigens in colorectal carcinoma, i.e. they are prominently overexpressed against all investigated healthy tissues, are printed in bold.

A comprehensive HLA ligand analysis of these CCA specimens has not been performed. However, peptides for some of the overexpressed antigens are known from the literature or from our own unpublished studies and are listed in Table 2.2.1.1.

Table 2.2.1.1. Potential tumor associated antigens in colorectal carcinomas. Genes had to be at least 4-fold overexpressed in tumor cells compared with autologous normal epithelial cells in at least 2 samples. Additionally, overexpression against a reference kidney sample was required. Genes displaying a promising expression profile against a larger number of healthy tissues are printed in bold. Already known HLA class I ligands or T cell epitopes are shown together with the HLA allele they bind to.

Probeset ID	Entrez Gene ID	Gene Symbol	Gene Title	Sequence	HLA
Overexpressed in all 4 CCAs					
202404_s_at	1278	COL1A2	collagen, type I, alpha 2		
201438_at	1293	COL6A3	collagen, type VI, alpha 3		
210445_at	2172	FABP6	fatty acid binding protein 6, ileal (gastrotropin)		
212942_s_at	57214	**KIAA1199**	**KIAA1199 protein**		
203961_at	10529	NEBL	nebulette		
205174_s_at	25797	QPCT	glutaminyl-peptide cyclotransferase (glutaminyl cyclase)		
205890_s_at	10537	**UBD**	**ubiquitin D**	**DANPYDSVKKI**	HLA-B*5101
				NPYDSVKKI	HLA-B*51
Overexpressed in 3 of 4 CCAs					
219555_s_at	55839	BM039	uncharacterized bone marrow protein BM039		
205476_at	6364	**CCL20**	**chemokine (C-C motif) ligand 20**		
204490_s_at	960	**CD44**	**CD44 antigen (homing function and Indian blood group system)**		
211657_at	4680	**CEACAM6**	**carcinoembryonic antigen-related cell adhesion molecule 6**		
201111_at	1434	**CSE1L**	**CSE1 chromosome segregation 1-like (yeast)**		
209774_x_at	2920	**CXCL2**	**chemokine (C-X-C motif) ligand 2**		
219787_s_at	1894	**ECT2**	**epithelial cell transforming sequence 2 oncogene**		
1438_at	2049	EPHB3	EphB3		
219118_at	51303	FKBP11	FK506 binding protein 11, 19 kDa		
40284_at	3170	**FOXA2**	**forkhead box A2**		
218507_at	29923	**HIG2**	**hypoxia-inducible protein 2**		
222062_at	9466	IL27RA	interleukin 27 receptor, alpha		
212792_at	23333	KIAA0877	KIAA0877 protein	IYTKIMDLI	HLA-A*24
36711_at	23764	MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)		
201930_at	4175	**MCM6**	**MCM6 minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)**		
208698_s_at	4841	NONO	non-POU domain containing, octamer-binding		
204351_at	6286	S100P	S100 calcium binding protein P		
200832_s_at	6319	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	SSYTTTTTI	HLA-B*1516
				ITAPPSRVL	HLA-A*02
				SPRENILVSL	HLA-B*07
				SPRENILVSL	HLA-B*0702
205009_at	7031	**TFF1**	**trefoil factor 1 (breast cancer, estrogen-inducible sequence expressed in)**		
210052_s_at	22974	**TPX2**	**TPX2, microtubule-associated protein homolog (Xenopus laevis)**		
Overexpressed in 2 of 4 CCAs					
201629_s_at	52	ACP1	acid phosphatase 1, soluble		
207158_at	339	**APOBEC1**	**apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1**		
205239_at	374	**AREG**	**amphiregulin (schwannoma-derived growth factor)**		
202095_s_at	332	**BIRC5**	**baculoviral IAP repeat-containing 5 (survivin)**	**ELTLGEFLKL**	HLA-A*0201
218542_at	55165	**C10orf3**	**chromosome 10 open reading frame 3**		
219857_at	79949	**C10orf81**	**chromosome 10 open reading frame 81**		
219099_at	57103	C12orf5	chromosome 12 open reading frame 5		
214710_s_at	891	**CCNB1**	**cyclin B1**	**FAFVTDNTY**	HLA-B*4601
				GEVDVEHT	HLA-B*40
200953_s_at	894	CCND2	cyclin D2	ATDFKFAMY	HLA-A*01
				ETSPLTAEKL	HLA-A*6601
203213_at	983	**CDC2**	**cell division cycle 2, G1 to S and G2 to M**		

Probeset ID	Entrez Gene ID	Gene Symbol	Gene Title	Sequence	HLA
209714_s_at	1033	**CDKN3**	**cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)**		
212501_at	1051	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta		
204170_s_at	1164	CKS2	CDC28 protein kinase regulatory subunit 2	KYFDEHYEY	HLA-C
218898_at	79850	CT120	membrane protein expressed in epithelial-like lung adenocarcinoma		
220890_s_at	51202	DDX47	DEAD (Asp-Glu-Ala-Asp) box polypeptide 47	YYIFIPSKF	HLA-A*24
201478_s_at	1736	DKC1	dyskeratosis congenita 1, dyskerin		
203693_s_at	1871	**E2F3**	**E2F transcription factor 3**		
201718_s_at	2037	**EPB41L2**	**erythrocyte membrane protein band 4.1-like 2**		
211048_s_at	9601	ERP70	protein disulfide isomerase related protein (calcium-binding protein, intestinal-related)		
219253_at	79134	FAM11B	family with sequence similarity 11, member B		
218802_at	55013	FLJ20647	hypothetical protein FLJ20647		
212858_at	124222	FLJ30002	hypothetical protein FLJ30002		
203988_s_at	2530	FUT8	fucosyltransferase 8 (alpha (1,6) fucosyltransferase)		
205240_at	29899	**GPSM2**	**G-protein signalling modulator 2 (AGS3-like, C. elegans)**		
205436_s_at	3014	H2AFX	H2A histone family, member X		
203744_at	3149	HMGB3	high-mobility group box 3		
218936_s_at	29080	HSPC128	HSPC128 protein		
211968_s_at	3320	HSPCA	"heat shock 90kDa protein 1, alpha"	AEDKENYKKF RRIKEIVKKH RRIKEIVKK KEKYIDQEEL	HLA-B*4403 HLA-B*2705 HLA-B*2705 HLA-A*2902
214022_s_at	8519	IFITM1	interferon induced transmembrane protein 1 (9-27)	TSVPDHVVW	HLA-B*5702
201892_s_at	3615	**IMPDH2**	**IMP (inosine monophosphate) dehydrogenase 2**		
219545_at	65987	KCTD14	potassium channel tetramerisation domain containing 14		
202503_s_at	9768	**KIAA0101**	**KIAA0101 gene product**		
218755_at	10112	**KIF20A**	**kinesin family member 20A**		
218355_at	24137	**KIF4A**	**kinesin family member 4A**		
212098_at	151162	LOC151162	hypothetical protein LOC151162		
212850_s_at	4038	**LRP4**	**low density lipoprotein receptor-related protein 4**		
203362_s_at	4085	MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)		
202107_s_at	4171	**MCM2**	**MCM2 minichromosome maintenance deficient 2, mitotin (S. cerevisiae)**		
222037_at	4173	**MCM4**	**MCM4 minichromosome maintenance deficient 4 (S. cerevisiae)**	**HSTIMPRL** **AHSTIMPRL** **DEFKIGELF**	HLA-B*5201 HLA-B*1510 HLA-B*18
204825_at	9833	**MELK**	**maternal embryonic leucine zipper kinase**		
222216_s_at	63875	MRPL17	mitochondrial ribosomal protein L17		
202911_at	2956	**MSH6**	**mutS homolog 6 (E. coli)**		
204766_s_at	4521	NUDT1	nudix (nucleoside diphosphate linked moiety X)-type motif 1		
215823_x_at	26986	**PABPC1**	**poly(A) binding protein, cytoplasmic 1**	**TRYQGVNLY**	HLA-B*2705
215157_x_at	5042	PABPC3	poly(A) binding protein, cytoplasmic 3		
200737_at	5230	**PGK1**	**phosphoglycerate kinase 1**		
205281_s_at	5277	PIGA	phosphatidylinositol glycan, class A (paroxysmal nocturnal hemoglobinuria)		
212371_at	51029	PNAS-4	CGI-146 protein		
209048_s_at	23613	**PRKCBP1**	**protein kinase C binding protein 1**		
212444_at	9052	RAI3	retinoic acid induced 3		
201890_at	6241	**RRM2**	**ribonucleotide reductase M2 polypeptide**	**REIIINAV**	HLA-B*4006
200660_at	6282	S100A11	S100 calcium binding protein A11 (calgizzarin)		
204900_x_at	8819	SAP30	sin3-associated polypeptide, 30kDa		

Probeset ID	Entrez Gene ID	Gene Symbol	Gene Title	Sequence	HLA
200631_s_at	6418	SET	SET translocation (myeloid leukemia-associated)	ELIAKIPNFW	HLA-A*25
219493_at	79801	SHCBP1	likely ortholog of mouse Shc SH2-domain binding protein 1		
203625_x_at	6502	SKP2	S-phase kinase-associated protein 2 (p45)		
209921_at	23657	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11		
204240_s_at	10592	SMC2L1	SMC2 structural maintenance of chromosomes 2-like 1 (yeast)		
202935_s_at	6662	SOX9	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	YTDHQNSSSYY	HLA-A*01
204092_s_at	6790	**STK6**	**serine/threonine kinase 6**		
200916_at	8407	TAGLN2	transgelin 2	YGMPRQIL	HLA-B*08
204045_at	9338	TCEAL1	transcription elongation factor A (SII)-like 1		
204033_at	9319	**TRIP13**	**thyroid hormone receptor interactor 13**		
202779_s_at	27338	UBE2S	ubiquitin-conjugating enzyme E2S	YAARARL	HLA-B*5802
221514_at	10813	**UTP14A**	**UTP14, U3 small nucleolar ribonucleoprotein, homolog A (yeast)**		
208648_at	7415	VCP	valosin-containing protein		
212533_at	7465	WEE1	WEE1 homolog (S. pombe)		

KIAA1199 is strongly overexpressed in all four CCA tumors and displays only low levels in the normal tissues for which data were available (Figure 2.2.1.4a). Additionally, this gene is annotated in the Entrez Gene database (http://www.ncbi.nlm.nih.gov/entrez) as "colon cancer secreted protein", even though no reference can be found for this statement in the literature. These circumstances would make KIAA1199 an ideal antigen for an immunotherapy of colorectal carcinoma. However, no HLA ligands are known so far for this gene. Unfortunately, these results are a prime example for the dangers inherent to this approach: KIAA1199 is specifically expressed in the inner ear and plays an important role in hearing [11]. Therefore, generating an immune response against peptides from this protein bears the risk of autoimmune reactions against healthy cells in the ear. In fact, autoimmunity in the inner ear is a well-established phenomenon with clinical manifestations [12, 13]. This example shows how important it is to include all available information in the selection process of overexpressed tumor antigens. However, for many genes not much is known so far and gene expression data remain as the only source of information. Unfortunately, gene expression data can never be specific enough to exclude expression in important cell types other than tumor cells. In bulk tissue samples, high expression in rare cells may be diluted to insignificant levels by the majority of irrelevant cells. Even if all different cell types in the body were accessible separately for gene expression analysis, variations in

different individuals would nevertheless be impossible to assess. Altogether, the selection process of overexpressed tumor antigens might be error-prone and the results should be critically scrutinized.

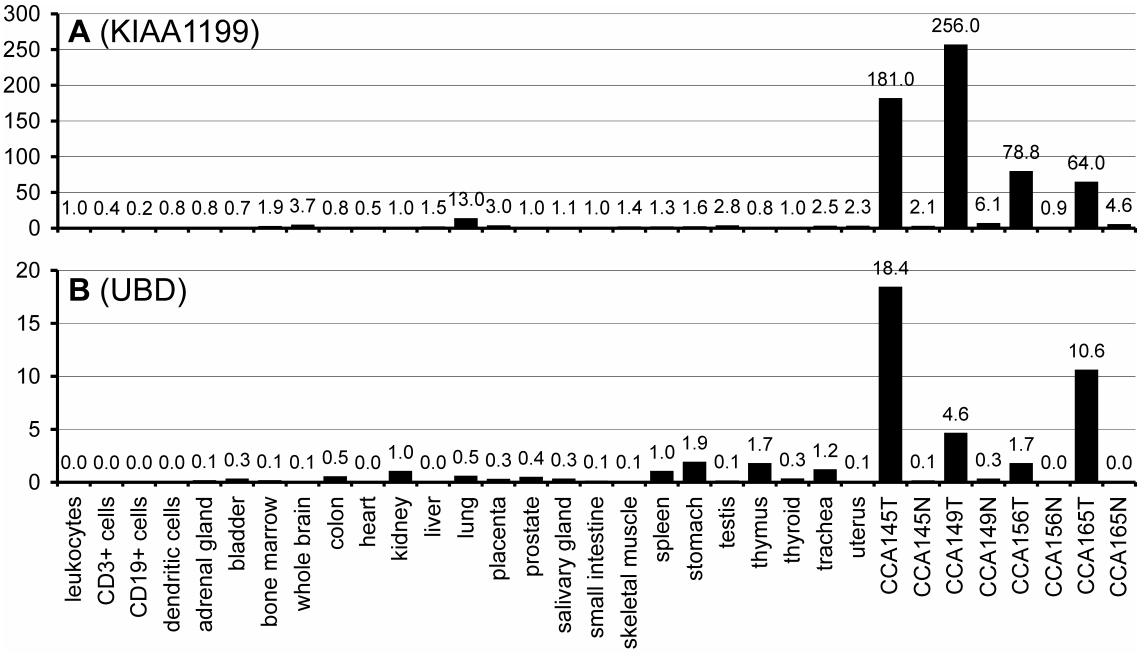

Figure 2.2.1.4. mRNA expression profiles of (A) KIAA1199 (B) ubiquitin D (UBD). Both genes are strongly overexpressed in all four CCAs compared with autologous normal colon samples and also overexpressed against other normal tissues.

Another example for a gene overexpressed in all four CCAs with a promising expression profile is ubiquitin D (UBD or FAT10). In this case, however, prominent overexpression against all normal tissues is only found for CCA145 and CCA165 (Figure 2.2.1.4b). For these patients though, UBD would likely be an attractive target antigen. It is encoded in the MHC class I region and inducible by IFN-γ [14]. Apart from its implications in protein degradation [15], UBD has been reported to be overexpressed in different cancers [16-18]. It might be involved in tumorigenesis or growth regulation through its binding to the mitotic spindle checkpoint protein MAD2 [14, 17]. Thus, expression data supported by possible mechanistic hints suggest UBD as a valuable target antigen. Interestingly, our own studies revealed a frequent overexpression also in renal cell carcinoma (see Figure 2.1.1.5b and [18]), suggesting that UBD might be a rather universal target antigen suitable for different tumor species. To test this antigen in peptide-based vaccinations, it would be desirable to identify other naturally presented HLA ligands for more frequent alleles apart from the two known HLA-B*51 binding peptides known so far (Table 2.2.1.1).

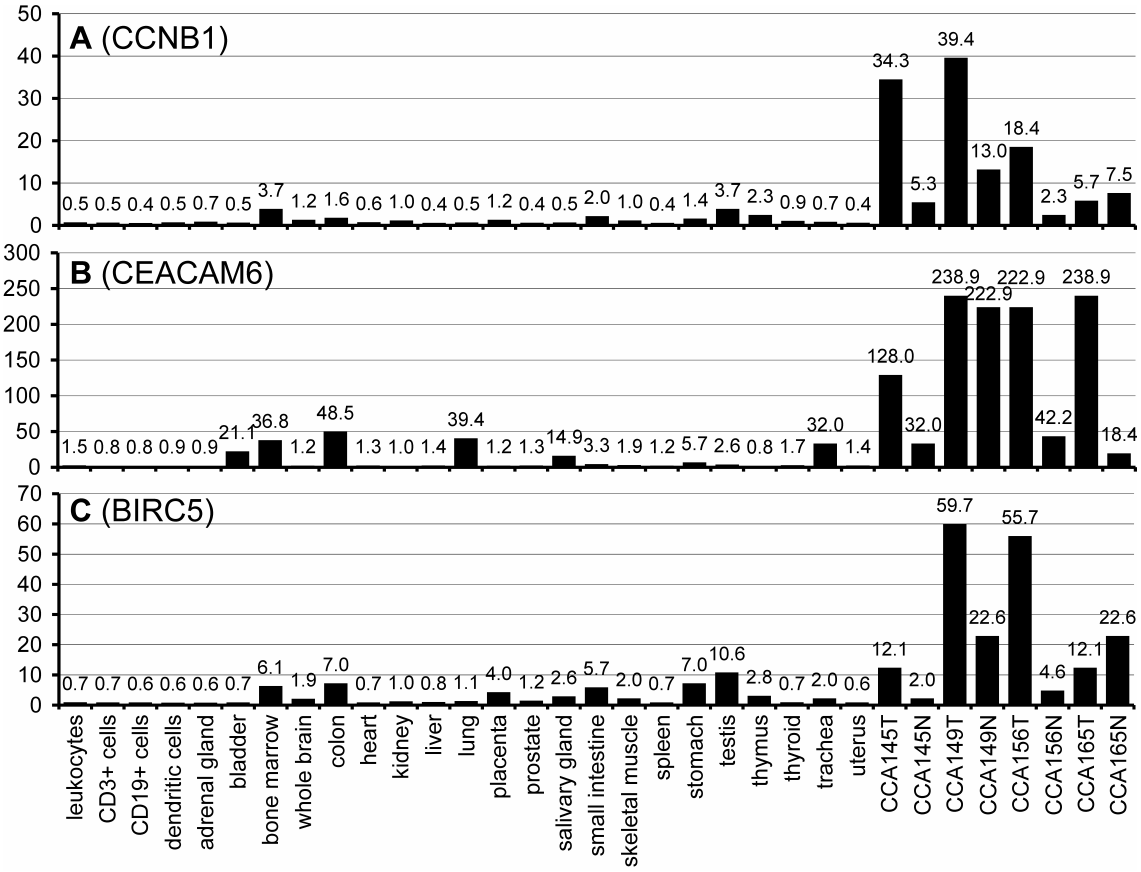

Figure 2.2.1.5. mRNA expression profiles of (A) cyclin B1 (CCNB1) (B) carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) (C) survivin (BIRC5). All three genes are strongly overexpressed in some CCAs compared with autologous normal colon samples and also upregulated against other normal tissues.

Besides UBD, other proteins involved in cell cycle regulation were upregulated as well in some CCA samples. Cyclin B1 (CCNB1, Figure 2.2.1.5a) is an example for which HLA ligands are known, albeit again for rather rare alleles (Table 2.2.1.1). This gene has been known for its implications in colorectal carcinogenesis before [19]. Involvement in tumorigenesis also applies to other potential target antigens related to cell cycle regulation, like cyclin-dependent kinase inhibitor 3 (CDKN3 or KAP) [20] or cell division cycle 2 (CDC2 or CDK1) [21, 22]. Interestingly, CDC2 plays an important role in the regulation of a pre-initiation complex for DNA replication consisting amongst others of MCM2, MCM4, and MCM6 [23, 24] - proteins which show up as interesting targets in Table 2.2.1.1 as well and have been described as cancer associated [25, 26]. Furthermore, MCM expression is regulated by the E2F transcription factor family [27, 28], whose member E2F3 is also a candidate target antigen in Table 2.2.1.1 [29]. Altogether, these interconnected target genes give a hint to a regulatory network which might be necessary for tumor growth and could be exploited for an anti-tumor therapy.

A well-established source of tumor antigens is the carcinoembryonic antigen (CEA) family [30-33]. In this study, CEACAM6 appeared as a promising target (Figure 2.2.1.5b). It has been suggested as tumor antigen before [34] and shows a high overexpression in all CCA samples as compared with healthy tissues. However, for CCA149 the expression in normal cells is equally high. This might be explained by the assumption that the "normal" cells in this case already showed some signs of transformation, because upregulation of CEACAM6 expression in hyperplastic polyps and early adenomas represents one of the earliest observable molecular events leading to colorectal tumors [35].

Finally, survivin (BIRC5) is another example of a fetally expressed gene which is well-established as tumor antigen in different cancers [36-38] and which was identified as a potential antigen for some CCAs (Figure 2.2.1.5c). For this antigen, a naturally processed HLA-A*02 binding peptide is known (Table 2.2.1.1, [39]), enabling a peptide-based immunotherapy of a considerable proportion of the population against different tumors including CCA with this antigen.

Conclusions

Comprehensive gene expression experiments on colon adenocarcinoma, either using laser microdissected cells or bulk tissue, are not new [40-46]. However, these studies mainly aim at the identification of marker genes or expression patterns for diagnostic purposes, i.e. tumor staging and substaging or classification according to susceptibility to different therapies. In contrast, this chapter focuses on therapeutic target identification: it demonstrates the feasibility but also some caveats of gene expression analysis in colon carcinoma for the detection of overexpression-based tumor antigens. Even though we have not yet performed comprehensive integrated gene expression analysis and HLA ligand identification for CCA as we have done for renal cell carcinoma (chapter 2.1.1), the results described here indicate that it is possible to successfully apply similar techniques to a more heterogeneous and therefore technically more challenging tumor entity.

2.2.1.4 References

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2.2.2 Technical excursus: Moderate degradation does not preclude microarray analysis of small amounts of RNA.

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The author of this thesis designed and performed all experiments described herein.

2.2.2.1 Summary

Gene expression analysis by microarrays using small amounts of RNA is becoming more and more popular against the background of advances and increasing importance of small-sample acquisition methods like laser microdissection techniques. The quality of RNA preparations from such samples constitutes a frequent issue in this context. The aim of this study was to assess the impact of different extents of RNA degradation on the expression profile of the samples. We induced RNA degradation in human tumor and healthy tissue samples by endogeneous ribonucleases. Next, we amplified 20 ng total RNA degraded to different extents by two rounds of *in vitro* transcription and analyzed them using Affymetrix oligonucleotide microarrays. Expression differences for some genes were independently confirmed by real-time quantitative PCR. Our results suggest that gene expression profiles obtained from partially degraded RNA samples with still visible ribosomal bands exhibit a high degree of similarity compared to intact samples and that RNA samples of suboptimal quality might therefore still lead to meaningful results if employed carefully.

2.2.2.2 Introduction

Since the initial days of gene expression analysis by DNA microarrays this technique has become more and more a standard tool for many research groups in all areas of life sciences. The possibility of measuring the expression of thousands of genes simultaneously under different conditions has led to new

insights into the behavior of various biological systems [1] and has provided new opportunities with respect to diagnosis or classification of diseases [2].

Recent advances in RNA and cDNA amplification methods have enabled microarray studies on small samples right down to very few cells [3, 4]. One of the most commonly applied methods for small-sample amplification is based on a technique originally described by the group of Eberwine [5]. The basic steps involve reverse transcription of mRNA with an oligo-dT primer containing a promoter sequence for T7 RNA polymerase. Synthesis of double-stranded cDNA is followed by an *in vitro* transcription (IVT) reaction resulting in multiple copies of antisense RNA (aRNA) from each cDNA molecule. Variations of this protocol enable its repetition in a second or even more rounds and therefore qualify it for the synthesis of sufficient amounts of aRNA or cDNA for microarray analysis from only few nanograms of total RNA. Even though such IVT based methods have been widely used, especially in combination with laser microdissection [6-9], it has only recently been demonstrated by extensive evaluations that they can indeed be applied without introducing too much artificial bias [10-16].

One important issue in the context of small-sample microarray analysis in particular remains the quality of initial RNA preparations. Methods aimed at the acquisition of single cells, for example laser microdissection techniques, frequently require a variety of additional steps that bear the risk of RNA degradation. Another problem may arise in the case of research on human tissue samples. Periods of warm ischemia between surgery and sample processing pose a serious threat to RNA integrity. It is often very challenging to decrease such times to a minimum within the framework of clinical routine procedures. RNA quality may also be impaired in samples stored for a long time or under suboptimal conditions. However, retrospective studies using the microarray technology now available on long-term archived tissues might be extremely valuable for the detection of new prognostic markers and the development of new treatments for diseases. Therefore, while it is obvious that intact RNA constitutes the best representation of the natural state of the transcriptome, there are situations in which gene expression analysis even on partially degraded RNA may be desirable. Nevertheless, little is known about the possibility of obtaining reasonable microarray data from RNA samples with impaired quality [17]. A recent publication suggests an amplification method

based on random priming that is shown to work remarkably well for degraded samples [18]. The aim of this study was to test a well-established two-round IVT protocol on 20 ng of partially degraded RNA and to assess the impact of such RNA degradation on gene expression data in comparison with high quality RNA.

2.2.2.3 Materials and Methods

RNA Samples

Tissue samples from a renal cell carcinoma (RCC) patient were obtained from the Department of Urology, University of Tübingen. The local ethical committee approved this study and informed consent was obtained from the patient. Following nephrectomy, fragments of normal and malignant renal tissue were dissected, shock-frozen in liquid nitrogen, and stored at -80°C. For the isolation of RNA the fragments were ground by mortar and pestle under liquid nitrogen and the frozen powder was transferred immediately into TRIzol® reagent (Invitrogen, Karlsruhe, Germany). In order to obtain RNA degraded by endogenous ribonucleases (RNases), aliquots of powdered tissue were incubated in an equal volume of PBS at 22°C for different time periods. TRIzol was added to stop degradation. Samples were homogenized by being passed through a 25-gauge needle. Total RNA was isolated according to the manufacturer's instructions and quantified by UV absorbance at 260 nm. Quality control was performed using the RNA 6000 Pico LabChip® Kit with a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Real-time quantitative PCR

RNA samples of tumor and normal tissue at different degradation states were used to synthesize single-stranded cDNAs. Reverse transcription was carried out in a reaction volume of 20 µL containing 1 µg total RNA, either 120 ng random hexamer primer (Amersham, Freiburg, Germany) or 500 ng oligo-(dT)15 primer (MWG Biotech, Ebersberg, Germany), 0.5 mM dNTPs (Promega, Mannheim, Germany), 10 U RNasin® (Promega), 10 mM DTT, 200 U SuperScript™ II reverse transcriptase (Invitrogen) and the reaction buffer supplied with the enzyme. Negative control reactions were carried out for each

sample by replacing the enzyme with water. The mixture was incubated at 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min.

Real-time quantitative PCR (qPCR) was performed using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). SYBR® Green PCR Master Mix (Applied Biosystems) was used for PCR amplification and real-time detection of PCR products. Primers (MWG Biotech) specific for different genes were designed to have a melting temperature of 60°C and are shown in Table 2.2.2.1. PCR reactions were carried out in 20 µL with 300 nM of each primer and with the following temperature profile: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were amplified in duplicate. Formation of undesired side products during PCR that contribute to fluorescence was excluded by melting curve analysis after PCR.

Table 2.2.2.1. Real-time quantitative PCR primers

Gene[a]	RefSeq[b]	Forward Primer	Reverse Primer
EEF2	NM_001961	5′-TCAACGAGTCCTTTGGCTTC-3′	5′-CTGCTGTTGTCGAAGGGG-3′
NNMT	NM_006169	5′-TTGAAGGGAACAGAGTCAAGG-3′	5′-GTCACATCACACTTCAGCACC-3′
TMSB10	NM_021103	5′-CTGCCGACCAAAGAGACC-3′	5′-GTGGCTCGTGTCCATCTTG-3′
MET	NM_000245	5′-ACATTGAAATGCACAGTTGGTC-3′	5′-ACAGGATCCACATAGGAGAATG-3′
SARS	NM_006513	5′-ATGATGGACAAGGTGGAGTTT-3′	5′-TTTCTCAGGCACAGTGATGC-3′
AMACR	NM_014324	5′-GGTCATTGATGCAAATATGGTG-3′	5′-ATCCAACATGTTCTGTCCTCG-3′
HSD11B2	NM_000196	5′-ATCACCGGCTGTGACTCTG-3′	5′-AGGTACGCAGCTCGATGG-3′

[a]Official gene symbols are used as abbreviations.
[b]RefSeq refers to the National Center for Biotechnology Information (NCBI) Reference Sequence indentification numbers that designate the mRNA sequence that was used for primer design.

Expression differences between tumor and normal tissue samples for different genes were calculated from PCR amplification curves by relative quantification using the comparative threshold cycle (CT) method (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). The housekeeping gene eukaryotic translation elongation factor 2 (EEF2) was equally expressed in both, tumor and normal tissue according to microarray data and was therefore chosen as reference for normalizations. The comparative CT method may be used when PCR amplification efficiencies for target and reference primer pairs are similar and close to 1. This was verified by serially diluting cDNA samples, performing qPCR with the different primer pairs, and calculating the amplification efficiencies from the slope of the line obtained by plotting CT values versus the logarithm of relative cDNA concentrations.

Microarray Analysis

Expression analysis was performed using Affymetrix HG-U133A or HG-Focus oligonucleotide microarrays (Affymetrix, High Wycombe, UK). The Focus array contains a subset of approximately 8500 sequences from the larger U133A array. The following analyses are restricted to the sequences (= probesets) represented on both arrays. An overview of the samples hybridized to microarrays is given in Table 2.2.2.2.

Table 2.2.2.2. RNA samples hybridized to microarrays

Sample/ Array[a]	Source	Degradation State	Total RNA Used[b]	Microarray
TA-U	Tumor	A	15 µg	U133A
NA-U	Normal	A	15 µg	U133A
TA-F1	Tumor	A	20 ng	Focus
TA-F2	Tumor	A	20 ng	Focus
NA-F	Normal	A	20 ng	Focus
TB-F	Tumor	B	20 ng	Focus
NB-F	Normal	B	20 ng	Focus
TD-U	Tumor	D	20 ng	U133A
ND-U	Normal	D	20 ng	U133A

[a]The first two letters of each sample correspond to Figure 1, the letter after the dash designates the array type (U for U133A, F for Focus).
[b]15-µg RNA samples were prepared for microarray analysis according to the standard protocol, and 20-ng RNA samples were prepared for microarray analysis according to the two-round protocol.

Standard protocol

One array pair for tumor and normal RNA of the best quality that could be obtained from the tissue samples (quality A) was generated from 15 µg total RNA according to the Affymetrix eukaryotic sample and array processing standard protocol (http://www.affymetrix.com/support/downloads/manuals/expression_s2_manual.pdf) which is based on the *in vitro* transcription (IVT) method originally described in [5].

Two-round protocol

We followed the protocol described in detail by Baugh et al. [10] for two rounds of IVT starting with 20 ng total RNA at different degradation states. The first round corresponds to the Affymetrix standard protocol described above with some modifications: First strand cDNA synthesis was performed in a reaction volume of 2 µl, T4 gene 32 protein (USB, Cleveland, OH, USA) was added at a

concentration of 0.4 µg/µL to increase the processivity of the reverse transcriptase [10], and the temperature was elevated to 48°C to increase yields. After the second-strand synthesis, IVT was performed with non-biotinylated NTPs using the AmpliScribe™ T7 High Yield Transcription Kit (Epicentre) for 9 h at 42°C. First-round aRNA was cleaned using RNeasy spin columns (Qiagen, Hilden, Germany) and used to generate second-round cDNA in a random hexamer-primed reverse transcription. The second cDNA strand was synthesized using a T7-(dT)24 primer (MWG Biotech). Biotinylated aRNA was generated from this dscDNA for 8 h at 40°C using the BioArray™ HighYield™ RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY, USA).

Microarray Hybridization and Staining

All procedures were carried out according to the Affymetrix standard protocol. Biotinylated aRNA was cleaned by RNeasy columns (Qiagen), fragmented, and 15 µg were used to prepare the hybridization cocktail. After hybridization for 16 h, microarrays were washed and stained using the instrument's standard protocol for the particular array type. Staining was performed with Streptavidin-Phycoerythrin using antibody-mediated signal amplification.

Data Analysis

Scanned images were processed using the Microarray Analysis Suite 5.0 (MAS 5.0, Affymetrix). Scaling of signal intensities was performed for each array based on the average intensity of 100 probesets representing housekeeping genes which are supposed to be expressed at similar levels in different cell types and which were selected by Affymetrix (http://www.affymetrix.com/support/technical/mask_files.affx). Expression differences between tumor and normal samples were determined by baseline comparison algorithms provided by MAS 5.0. The normal sample was always defined as the baseline. Data were further processed using Microsoft Access and Excel.

2.2.2.4 Results and Discussion

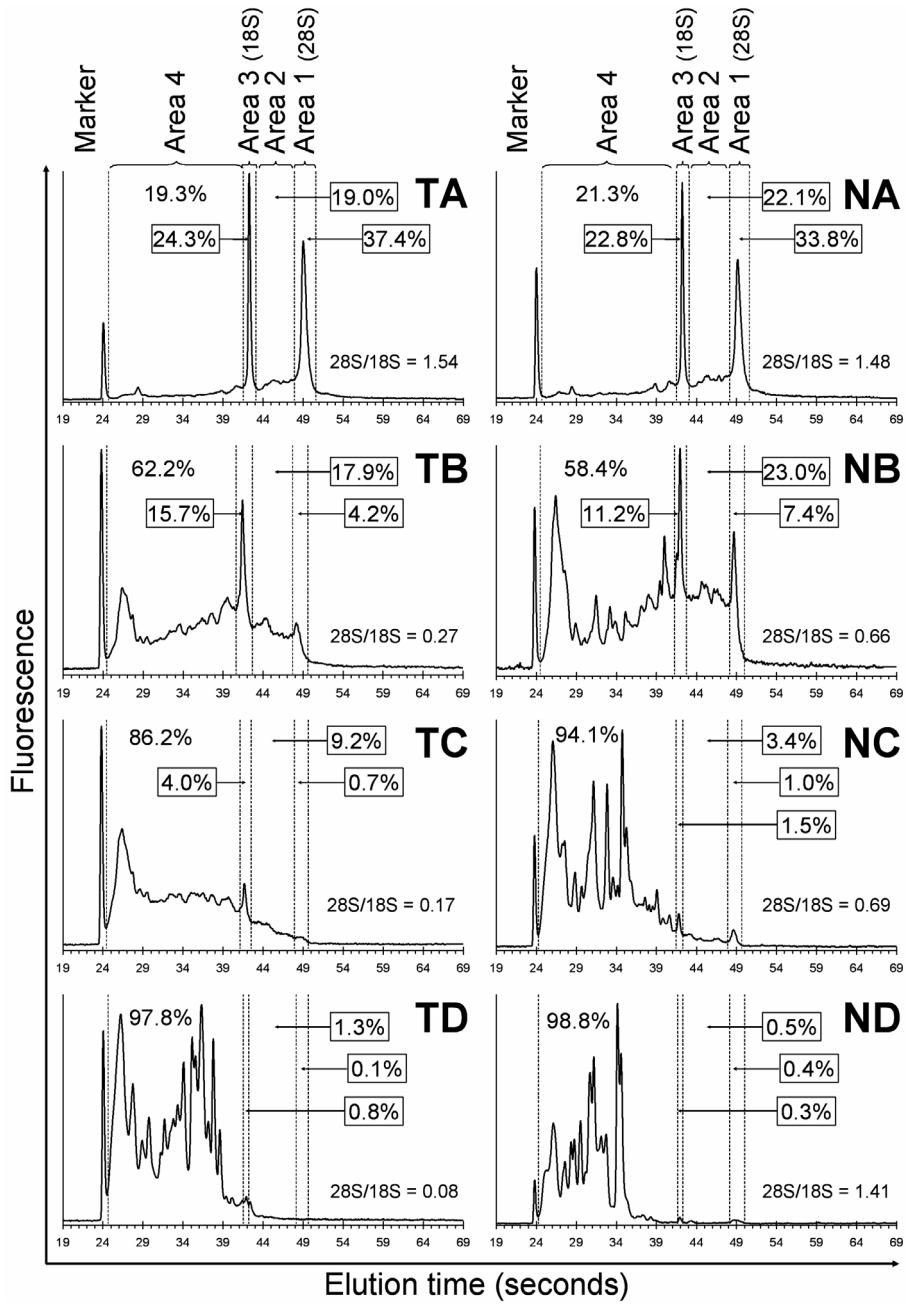

Figure 2.2.2.1. Electropherograms of RNA samples degraded to different extents. Analysis was performed using the RNA 6000 Pico LabChip Kit on a Bioanalyzer (Agilent). The left column shows RNA samples isolated from tumor (TA-TD), the right column from normal healthy tissue (NA-ND). Tissue sample were incubated at room temperature for the following time periods prior to RNA isolation: TA, NA: 0 min (freshly isolated); TB: 10 min; NB: 2 min; TC: 20 min; NC: 5 min; TD: 90 min; ND: 15 min. The letters A, B, C, and D were assigned to samples in the order of increasing degradation. Although degradation patterns vary considerably between tumor and normal samples, the same letter was chosen for samples of comparable quality. To assess the size distribution of the RNA fragments quantitatively, four adjacent areas were defined as indicated. For an approximate calculation of the RNA amount, each intensity value (after subtraction of the baseline) was multiplied by the time between the data points and the results were summed up for each area. Percentages given in the figure indicate RNA amounts in each area in relation to the combined amount in all four areas. 28S/18S ratios were calculated from the respective percentages.

RNA degradation

Powdered frozen tissue samples from a renal cell carcinoma patient were thawed and then incubated at room temperature in order to obtain RNA degraded by ribonucleases present in the tissue itself. This should lead to degradation patterns representative for naturally degraded samples. The quality of total RNA preparations is generally assessed according to the clear visibility of ribosomal 28S and 18S bands at a ratio of 2:1 and the absence of any other nucleic acid species after separation by electrophoresis. We performed capillary electrophoresis of high quality and degraded samples using a Bioanalyzer (Figure 2.2.2.1). The incubation times necessary to reach a certain degradation state were considerably longer for the tumor as compared to the normal tissue samples. This might reflect either decreased RNase activity in this tumor tissue or some other mechanism of RNA stabilization in the tumor. The observation cannot be explained by an overexpression of ribonucleases at the mRNA level in normal tissue as indicated by the microarray data of these samples (data not shown).

For a quantitative description of the RNA degradation state we found the 28S/18S ratio to be of no practical value. It should only be used as long as there are no prominent degradation products. Otherwise its sole use for the characterization of the sample can be misleading. This is especially apparent for the heavily degraded sample ND (Figure 2.2.2.1) that still has a 28S/18S ratio of 1.4. Therefore, we decided to include two additional parameters in the assessment: the amount of degradation products between the ribosomal peaks and the amount below the 18S peak. According to this model, the RNA samples that we refer to as “moderately degraded” (TB and NB, Figure 2.2.2.1) can be characterized as follows: The 28S peak still accounts for approx. 5% of the overall amount of RNA. 20% of fragmented RNA is located between the 28S and 18S rRNA whereas a bit more than 10% is found in the 18S area. Most importantly, about 60% of the overall RNA has a fragment size smaller than 18S rRNA. We would regard a sample that has more than 65% RNA in this area and less than 4% in the 28S area as heavily degraded.

Relative expression levels measured by qPCR are conserved in partially degraded RNA.

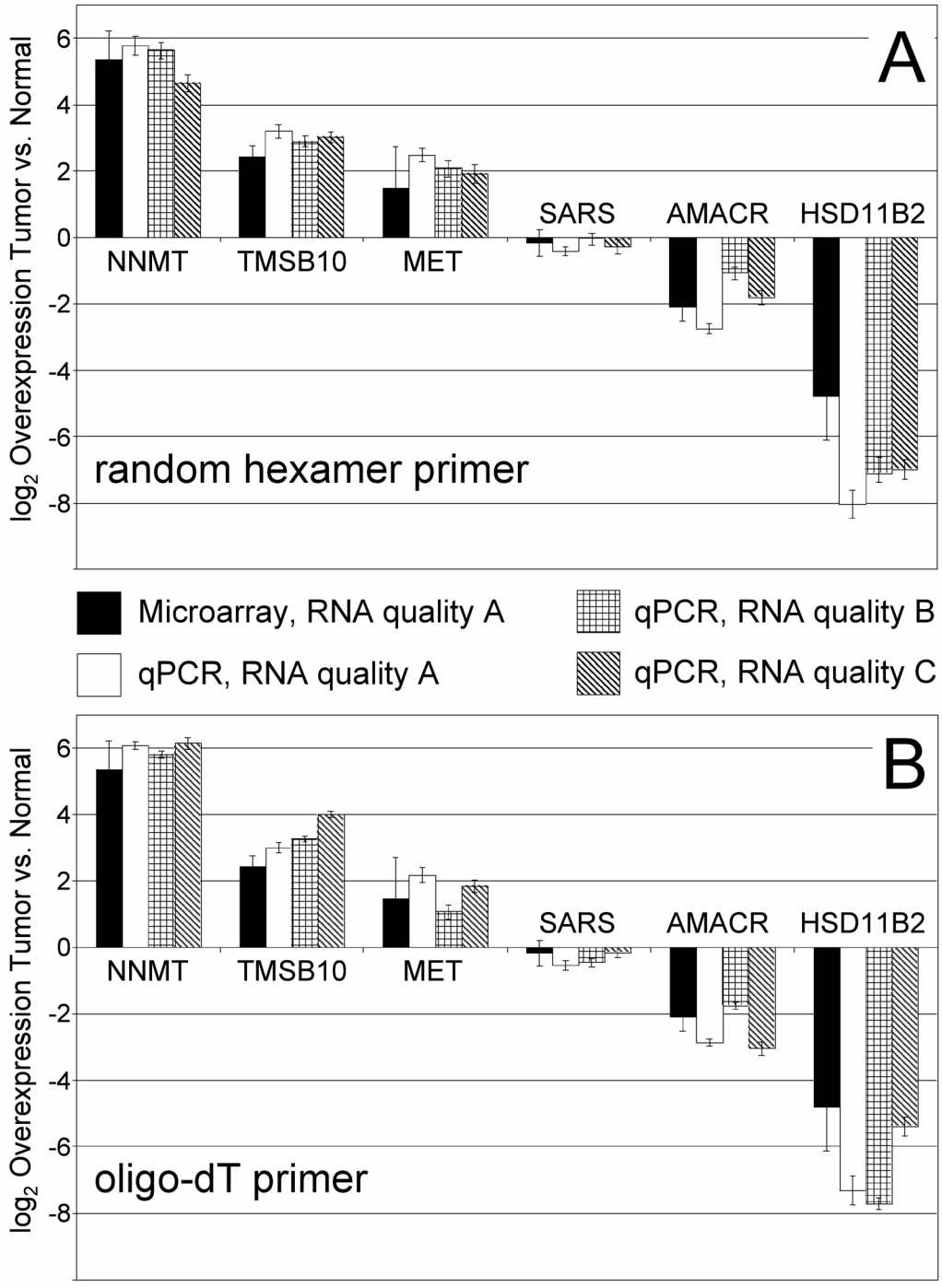

Figure 2.2.2.2. Differential expression of selected genes between tumor and healthy normal tissue measured by real-time quantitative PCR and microarrays. Negative values indicate overexpression in normal tissue compared with the tumor. For panel A, cDNA was synthesized using random hexamer primers, for panel B, an oligo-dT primer was used. The first bar for each gene (Microarray, RNA quality A) indicates the expression difference measured by microarray using the samples TA-U and NA-U (Table 2.2.2.2). The following three bars show qPCR measurements using cDNAs from the samples with degradation states A, B and C (Figure 2.2.2.1). qPCR values are normalized to the endogenous reference gene EEF2. Error bars for qPCR values are standard deviations. Error bars for microarray values indicate 95% confidence intervals as calculated by the Affymetrix MAS 5.0 software.

qPCR was performed on cDNA synthesized directly from intact or degraded RNA samples without previous amplification by IVT. Figure 2.2.2.2 shows expression differences between tumor and normal healthy tissue for six genes measured at different RNA degradation states A, B, and C as defined in Figure 2.2.2.1. qPCR measurements are frequently used as an independent method for the verification of microarray data. Comparison of the first two bars for each gene in Figure 2.2.2.2 shows a high concordance between the two methods. HSD11B2 was not clearly detected above background levels in the tumor sample. Therefore, the expression difference between tumor and normal samples for this gene rather reflects expression in normal tissue compared with the background of the particular method.

Expression differences obtained from degraded samples using random-primed cDNA corresponded considerably well to high-quality samples (Figure 2.2.2.2A). This is to be expected since random-priming captures all RNA fragments, and qPCR amplicons have a length in the range of only 100 base pairs (bp). Therefore fragmentation of RNA will result in a loss of the molecule for qPCR detection only if the break occurs within the short amplicon. This might be a rare event in only moderately degraded RNA. For the cDNA samples synthesized with oligo-dT primers only poly-A bearing 3' ends of mRNA molecules are reverse transcribed. In degraded samples all fragments missing the original 3' end are lost. Nevertheless, expression differences measured with oligo-dT-primed cDNA from degraded RNA (Figure 2.2.2.2B) still corresponded quite well to the values obtained from intact RNA. This might indicate that each individual mRNA species was affected quite uniformly by degradation in both tumor and normal tissue.

Moderate RNA degradation disturbs microarray results only slightly.

qPCR measurements with oligo-dT-primed cDNA indicated that the 3' ends of degraded mRNA were sufficient to obtain reasonable gene expression differences. This finding encouraged us to apply a protocol on degraded RNA that is capable of generating labeled target RNA for microarray analysis by two rounds of *in vitro* transcription from total RNA amounts in the low nanogram range. This protocol leads to a pronounced 3' bias of the resulting antisense RNA transcripts, especially in degraded samples. Our aim was to test the impact of RNA degradation on the measurement of differential gene expression

using Affymetrix microarrays that are designed to preferentially probe the 3' ends of transcripts. The two-round IVT protocol was applied to 20 ng samples of degradation states A, B and D (Figure 2.2.2.1). Samples TA and NA were also subjected to the standard one-round protocol using 15 µg RNA.

Table 2.2.2.3. Characteristics of microarray experiments

Sample/Array[a]	aRNA Yield[b] (µg)	Scale Factor	Genes Present[c] (%)	3′/5′ Ratio[d] (GAPDH)	3′/5′ Ratio[d] (β-Actin)
TA-U	67	0.86	52.2	0.91	1.08
NA-U	72	1.13	50.5	1.07	1.13
TA-F1	53	0.63	58.4	1.34	3.87
TA-F2	59	0.53	58.6	1.17	5.44
NA-F	69	0.68	56.1	1.91	6.04
TB-F	23	0.98	54.1	3.52	22.82
NB-F	41	1.07	55.6	2.79	9.04
TD-U	11	10.22	16.1	9.05	11.46
ND-U	24	2.01	36.5	6.94	13.58

[a]The first two letters of each sample correspond to Figure 1, the letter after the dash designates the array type (U for U133A, F for Focus).
[b]Antisense RNA (aRNA) yields refer to biotinylated aRNA. For TA-U and NA-U, only 50% of cDNAs were used for in vitro transcription (IVT). Equal amounts of 15 µg aRNA were hybridized to microarrays with the exception of TD-U and ND-U, for which only 11 µg were used.
[c]The percentage of genes detected as present refers to 8746 probesets represented on the Focus array.
[d]3′/5′ ratios are calculated using different probesets on the array that target the 3′ or 5′ end of the particular gene. Ratios greater than 1 indicate a loss of 5′ ends among aRNA transcripts.

Table 2.2.2.3 gives an overview of some general characteristics for each microarray. The two-round protocol applied to high quality RNA (TA-F1, TA-F2, NA-F) led to lower scale factors, slightly higher percentages of genes detected as present, and increased 3'/5' ratios if compared to the standard protocol for high quality RNA (TA-U, NA-U). These differences may be explained by the fact that 3'-biased target RNA was applied to a 3'-biased microarray. Moderate degradation (TB-F, NB-F) caused an increase in scale factors and 3'/5' ratios but only a slight decrease in the percentage of present genes, indicating that the sensitivity was largely unaffected by this kind of degradation. A pronounced loss in data quality became apparent for the most degraded samples (TD-U, ND-U).

In order to identify differentially expressed genes, microarrays from normal tissue samples were defined as the baseline and tumor samples were compared to them. Figure 2.2.2.3 shows numbers of genes identified as up- or downregulated in common between different pairs of tumor and normal arrays together with the corresponding percentages. The highest percentage of overlapping genes (88%) was found for replicate tumor arrays compared with the same normal array (TA-F1 vs. NA-F compared with TA-F2 vs. NA-F). This

replicate setting indicates the minimal extent of inevitable variation immanent in the sample preparation and measurement procedure. The comparison of those small sample replicate pairs with the standard protocol array pair of the same starting RNA (TA-U vs. NA-U) showed slightly decreased percentages (~ 85%) representing small differences between the two protocols. The partially degraded sample pair (TB-F vs. NB-F), however, demonstrated a clear loss in concordance with the data obtained from high quality samples. Whereas all high quality samples showed an average 86% overlap regardless of the protocol used for preparation, it decreased to an average of 71% if the partially degraded pair was compared to each of the high quality samples. For the completely degraded sample pair the absolute number of differentially expressed genes was markedly lower than for the high quality and partly degraded samples, which showed no differences in this parameter. This resulted in a strong decrease in the number of regulated genes identified in common with the other sample pairs. It is remarkable, that elevating or lowering the threshold for the genes to be considered for this approach does not noticeably change the results (data not shown). This indicates that the perturbation introduced by the degradation cannot be filtered out by focusing only on genes with strong expression differences.

	TA-U vs. NA-U	TA-F1 vs. NA-F	TA-F2 vs. NA-F	TB-F vs. NB-F	TD-U vs. ND-U
TA-U vs. NA-U	1405	85%	84%	73%	62%
TA-F1 vs. NA-F	1191	1712	88%	70%	65%
TA-F2 vs. NA-F	1175	1430	1626	70%	63%
TB-F vs. NB-F	1020	1199	1134	1715	62%
TD-U vs. ND-U	388	409	397	389	629

Figure 2.2.2.3. Differentially expressed genes identified in common between samples at different degradation states. Black cells show the number of genes that were identified as either increased or decreased between tumor and normal tissue by each particular pair of microarrays (Table 2.2.2.2). The lower left part of each table indicates the number of such up- and downregulated genes that were found in common between different microarray pairs. The grey shaded upper right part of the tables shows the corresponding percentages. The smaller number of regulated genes among the two array pairs constitutes the maximal number that could be detected in common and was therefore set as 100 percent. To be considered as differentially expressed, genes had to fulfill general criteria for up- or downregulation: an “increase” call together with a “present” call on the tumor array or a “decrease” call together with a “present” call on the normal array. Furthermore, a threshold criterion of twofold increase or decrease had to be met.

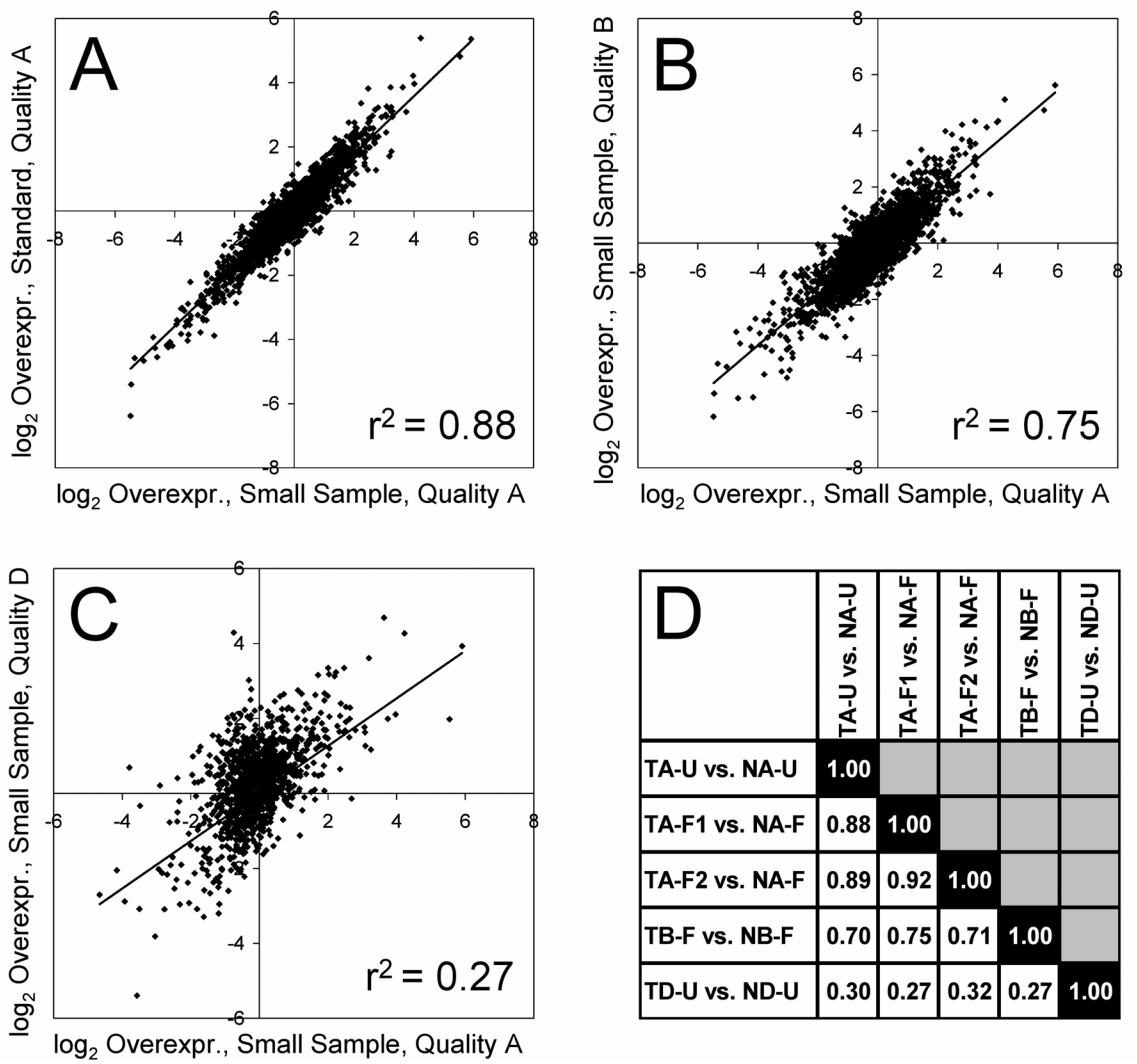

Figure 2.2.2.4. Correlation of overexpression measured at different degradation states. Expression differences between tumor and normal tissue were quantitatively determined by baseline comparisons using the MAS 5.0 software for different pairs of tumor and normal arrays. The $\log_2$ values for differential expression obtained by each array pair were compared with the other pairs: **(A)** Comparison of the standard labeling protocol using high quality RNA (TA-U vs. NA-U, Table 2.2.2.2) with the small sample protocol applied to the same RNA (TA-F1 vs. NA-F); **(B)** Partly degraded RNA (TB-F vs. NB-F) compared with high quality RNA (TA-F1 vs. NA-F); **(C)** Completely degraded RNA (TD-U vs. ND-U) compared with high quality RNA (TA-F1 vs. NA-F); **(D)** Correlation coefficients r^2 for all comparisons between array pairs.
This analysis was restricted to 3288 genes that were detected as present on all microarrays of degradation state A and B. For comparisons that involve the most degraded samples D the analysis had to be further restricted to 1129 genes also present on those arrays. This pre-selection of genes is necessary in order to avoid measuring differential expression against background levels which would result in meaningless numbers inappropriate for measuring correlation in this way.

In addition to this kind of analysis, we wanted to obtain a more comprehensive picture by analyzing differential expression in a more quantitative way. Figure 2.2.2.4 shows expression differences for a large set of genes measured by two different array pairs at a time. Each data point indicates the $\log_2$ fold-change values for one gene measured by each of the compared array pairs. Correlation coefficients for all comparisons of tumor vs. normal pairs are given in Figure

2.2.2.4D. As expected, the highest r^2 value (0.92) was achieved by comparing semi-replicate array pairs (TA-F1 vs. NA-F and TA-F2 vs. NA-F). Comparison of these pairs with the standard protocol pair of the same RNA (TA-U vs. NA-U) resulted in r^2 values close to the former (0.88 and 0.89). The correlation coefficients were somewhat decreased when the partially degraded sample pair was compared to the high quality pairs (0.70 to 0.75). However, this result still indicates a distinct tendency of this sample pair to reflect the overall expression pattern observed with high quality samples. In contrast, the most degraded sample pair showed only a weak correlation with the other samples which makes a trustworthy analysis impossible.

Taken together, our methods for analyzing the microarray data indicated a certain decrease in data quality for the moderately degraded samples. A distinct loss of 5' ends of transcripts was evident from the 3'/5' ratios measured by microarrays. Nevertheless, a pronounced decrease in the number of genes detected or differentially expressed did not result from this loss. However, moderate RNA degradation introduced a certain variation into differential expression data. Even though comparable in size, sets of up- or downregulated genes between partially degraded and high quality samples showed a smaller overlap than was observed among high quality samples alone. Furthermore, correlations of quantitative expression differences determined between partially degraded and intact samples dropped behind correlations measured with intact RNA samples alone. Data obtained with the most degraded samples demonstrated such a low overall quality and correlation with the initial samples that they could not be reasonably considered for analysis.

Genes identified using this approach should be validated with special care. While possible perturbations introduced by RNA degradation itself cannot be accounted for, at least possible artificial influences caused by the 3' bias of the two-round IVT procedure can be excluded by qPCR measurements using random-primed cDNA, which contains all mRNA fragments of the degraded sample.

In conclusion, while all efforts should be made to obtain high quality RNA samples that reflect the natural state most reliably, moderately degraded samples with a degradation signature similar to our samples TB and NB (Figure 2.2.2.1) – 5% of the RNA in the 28S rRNA area and no more than 60% with fragment sizes smaller than 18S rRNA - may still lead to a reasonable

expression profile. 20 ng of such degraded RNA are sufficient for microarray analysis after two rounds of linear amplification by *in vitro* transcription. Expression differences measured with this method and verified by quantitative real-time PCR are similar to those obtained from high quality samples.

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2.2.2.6 References

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2.3 Microarrays in a more fundamental immunological context with implications for tumor immunology

2.3.1 Autophagy promotes MHC class II presentation of peptides from intracellular source proteins

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2.3.1.1 Abstract

MHC-peptide complexes mediate key functions in adaptive immunity. In a classical view, MHC-I molecules present peptides from intracellular source proteins, whereas MHC-II molecules present antigenic peptides from exogenous and membrane proteins. Nevertheless, substantial crosstalk between these two pathways has been observed. We investigated the influence of autophagy on the MHC-II ligandome and demonstrate that peptide presentation is altered considerably upon induction of autophagy. The presentation of peptides from intracellular and lysosomal source proteins was strongly increased on MHC-II in contrast to peptides from membrane and secreted proteins. In addition, autophagy influenced the MHC-II antigen processing machinery. Our study illustrates a profound influence of autophagy on the class II peptide repertoire and suggests that this has implications for the regulation of CD4+ T-cell mediated processes.

2.3.1.2 Introduction

Peptides of foreign and self proteins are presented on major histocompatibility complex class I (MHC-I) and class II (MHC-II) molecules at the cell surface and can be recognized by CD8+ and CD4+ T lymphocytes, respectively [1, 2]. From a classical point of view, MHC-I molecules present antigenic peptides derived from intracellular proteins, whereas MHC-II molecules do so for exogenous and membrane proteins [3]. This phenomenon is reflected in the two major cellular breakdown pathways for proteins: proteasomal degradation, particularly relevant to the generation of MHC-I peptides [4], and degradation by the endosomal/lysosomal system, which is responsible for the processing of MHC-II peptides [5]. However, the separation of these distinct pools of source proteins is less stringent than originally believed. It is now well-established that MHC-I molecules are able to present peptides derived from exogenous antigens (Ag) by a process known as cross presentation [6]. On the other hand, intracellular proteins can be presented by MHC-II molecules [7, 8] even though the underlying processes are less clear. It has been recently shown that peptides from cytosolic model proteins can be presented on MHC-II molecules via autophagy [9-11]. Autophagy plays a role in the endosomal/lysosomal degradation pathway and is responsible for feeding intracellular components into this pathway. It is thought to be required for normal turnover of cellular components, particularly in response to starvation [12]. Against this background, we hypothesized that autophagy might mediate MHC-II presentation of intracellular Ag – meaning the contents of a cell contained within the plasma membrane, excluding large vacuoles and secretory or ingested material (Gene Ontology classifications) – in general. Therefore, we performed a detailed characterization of the MHC-II ligand repertoire (ligandome) presented at the cell surface under normal conditions and after increased autophagy, leading to a comprehensive overall picture of changes in peptide processing and presentation.

2.3.1.3 Materials and Methods

Cells and antibodies

The human B-lymphoblastoid cell lines Awells (IHW-No. 9090; HLA-DRB1*0401, HLA-DRB4*0101) and Awells-Ii-LGALS2 (Awells transfected with a fusion gene encoding the 80 N-terminal amino acids of Ii and LGALS2) were

maintained at 37°C in DMEM (C.C.Pro, Neustadt, Germany) containing 10 % FCS (Pan, Aidenbach, Germany) and supplemented with 2 mM L-glutamine (BioWhittaker, Verviers, Belgium), 100 U/ml penicillin, and 100 µg/ml streptomycin (BioWhittaker). During induction of autophagy, cells were kept in Hank's Balanced Salt Solution (HBSS). For comparative ligand analysis it was crucial to maintain the cells at a density of 0.2×10^6 cells/ml. If cells were kept at higher densities, culture medium was rapidly exhausted leading to high basal autophagy levels. For autophagy inhibition, cells were kept in DMEM or HBSS supplemented with 10 mM 3-methyladenine (Sigma-Aldrich, Steinheim, Germany). Dead cells were generated by 3 rounds of freezing in liquid nitrogen and thawing at 37°C. After this procedure no live cells could be detected by light microscopy. The antibody L243 (anti–HLA-DR) [13] was purified from hybridoma culture supernatants using protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). Antibodies used in flow cytometry analysis were from PharMingen (San Diego, CA, USA).

Analysis of monodansylcadaverine (MDC) labeled vacuoles

Autophagic vacuoles were labeled with MDC and analyzed using either fluorescence microspcopy [14, 15] or fluorescence spectroscopy in cell lysates [15], essentially as described. Briefly, cells were incubated at 37°C for 10 min with 0.05 mM MDC and subsequently washed four times with PBS. Cells were either analyzed by live cell microscopy or lysed in 10 mM Tris-HCl, pH 8 containing 0.1% Triton X-100 for fluorescence spectroscopy. After lysis, remaining cellular debris was spun down.

Fluorescence Microscopy

Live cells were immediately analyzed at room temperature by epifluorescence microscopy on an inverted microscope (Axiovert 63W; Carl Zeiss, Jena, Germany) fitted with a 63 x 1.2 numerical aperture lens in eight-well chambered cover glasses (Nunc, Wiesbaden, Germany). Fluorescence emission and detection was performed with a filter system (excitation BP 365 nm, detection LP 397 nm, beam splitter FT 395 nm). Images were acquired with a Sensicam cooled 12-bit CCD camera (PCO Computer Optics, Kelheim, Germany) and processed using the program Axiovision 3.1 (Carl Zeiss).

Fluorescence Spectroscopy

MDC concentrations in whole cell lysates [15] were determined using an LS50B spectrofluorometer (Perkin-Elmer, Norwalk, CT, USA), with excitation at 380 nm and detection of emission at 525 nm. The protein content of the lysates was determined using a commercially available Bradford protein assay kit (Bio-Rad Laboratories, München, Germany).

Gene expression analysis by high-density oligonucleotide microarrays

Total RNA was isolated from Awells using Trizol (Invitrogen, Karlsruhe, Germany) followed by an RNeasy cleanup (QIAGEN, Hilden, Germany) after autophagy induction for 6 h and 24 h and from cells cultured in normal medium for the same times as controls. High RNA quality was ensured by a 2100 Bioanalyzer (Agilent, Waldbronn, Germany) assay using the RNA 6000 Pico LabChip Kit (Agilent). Gene expression analysis of the four RNA samples was performed by Affymetrix Human Genome U133 Plus 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA) according to the Affymetrix manual (http://www.affymetrix.com/support/technical/manual/ expression_manual.affx). Briefly, double-stranded cDNA was synthesized from 8 µg of total RNA using SuperScript RTII (Invitrogen) and the oligo-dT-T7 primer (MWG Biotech, Ebersberg, Germany) as described in the manual. *In vitro* transcription was performed with the BioArray™ High Yield™ RNA Transcript Labeling Kit (ENZO Diagnostics, Inc., Farmingdale, NY, USA), followed by cRNA fragmentation, hybridization, and staining with streptavidin-phycoerythrin and biotinylated anti-streptavidin antibody (Molecular Probes, Leiden, NL). Images were scanned with the Affymetrix GeneChip Scanner 3000 and data were analyzed with the GCOS software (Affymetrix) using default settings for all parameters.

Normalization was performed by scaling all four arrays based on the overall fluorescence intensity of each array. Scaling factors differed by no more than a factor of 1.2 and all other quality control parameters provided by the array indicated a high data quality. For each of the two time points a pairwise array comparison was calculated with the autophagy array as the experiment and the respective normal medium control array as the baseline.

In order to identify functional categories or pathways for which a higher proportion of genes were up- or downregulated compared with the overall proportion of regulated genes, the following analysis was performed: First,

genes were selected that were reproducibly up- or downregulated after 6 h and 24 h starvation according to the GCOS software. To be classed as “upregulated”, a gene had to fulfill the following requirements: "increase" in the change call algorithm, demonstrating "present" in the detection call algorithm for the autophagy array, and a $\log_2$ overexpression (signal log ratio, SLR) of at least 0.5 (approx. 1.4-fold overexpression). Downregulated genes had to show a "decrease", a "present" on the control array and an SLR equal to or smaller than -0.5. According to this, 1336 probe sets were reproducibly upregulated and 1680 downregulated. In a second step, these genes were analyzed using MAPPFinder [16] and EASE [17]. Both programs aim at the identification of overrepresented biological themes within lists of genes based on gene ontology (GO) categories. The 8-10% of GO terms with the best scores for overrepresentation were selected for each of the programs and for both, up- and downregulated genes. GO terms identified in common by both programs were further edited manually to avoid extensive redundancy among overlapping terms and to exclude terms that were too general to draw any conclusions from them. The remaining GO categories are shown in Table 2.3.1.3 and Table 2.3.1.4. In a second experiment, microarray analysis was repeated for freshly prepared batches of starved (24 h) and control cells. For the majority of genes shown in Table 2.3.1.3 and Table 2.3.1.4 the results in terms of up- or downregulation could be reproduced. Microarray data are available from the GEO repository (http://www.ncbi.nih.gov/geo/) with the accession no. "GSE2435".

Western blot

Cells/fractions lysed in NP-40/pH 7 lysis buffer (50 mM sodium acetate, 5 mM $MgCl_2$, 0.5% NP-40) were resolved by 12.5 % SDS-PAGE, transferred to PVDF membrane (Millipore, Bedford, MA, USA), blocked, and probed with appropriate dilutions of the respective primary antibody, followed by a secondary anti-rabbit IgG antibody coupled with peroxidase (Southern Biotech, Birmingham, AL, USA). An ECL detection Kit (Amersham Pharmacia, Freiburg, Germany) was used to visualize the Ab-reactive proteins. Anti-cathepsin polyclonal antisera were provided by E. Weber (University of Halle, Germany).

Affinity-labelling of active cysteine proteases and in vitro digestions

Crude endocytic fractions were generated by ultracentrifugation of postnuclear supernatants as described [18]. 5 µg total endocytic protein were incubated with reaction buffer (50 mM citrate/phosphate pH 5.0, 1 mM EDTA, 50 mM DTT) in the presence of DCG-0N, a derivative of DCG-04 that shows the same labelling characteristics [19] for 1 h at room temperature. Reactions were terminated by addition of SDS reducing sample buffer and immediate boiling. Samples were resolved by 12.5 % SDS-PAGE, then blotted on a PVDF-membrane and visualized using streptavidine HRP and the ECL-detection kit [20]. MBP_{83-99} digestions were performed as described [21].

Elution of MHC Class II bound Peptides

Frozen cell pellets (1×10^9 to 5.7×10^{10}cells) were processed as previously described [22] and peptides were isolated according to standard protocols [23] using 5 mg to 25 mg HLA-DR specific mAb L243 [13].

Molecular analysis of HLA-DR-eluted peptides

Peptides were separated by reversed-phase high performance liquid chromatography (HPLC, SMART system, µRPC C2/C18 SC 2.1/10; Amersham Pharmacia Biotech, Freiburg, Germany), and fractions were analyzed by MALDI-TOF mass spectrometry (MS) using a Bruker Reflex III mass spectrometer (Bruker Daltonik, Bremen, Germany). Peptides were further analyzed by nano-ESI (electrospray ionisation) MS/MS either on a Q-TOF mass spectrometer (Micromass, Manchester, United Kingdom) or on a QStar Pulsar i Qqoa Tof mass spectrometer (Applied Biosystems-MDS Sciex, Weiterstadt, Germany) as described [22, 23].

For comparative peptide analysis between peptides eluted from $1\text{-}3\times10^9$ control cells and $1\text{-}2\times10^9$ cells undergoing autophagy, peptides were analyzed by a reversed phase Ultimate LC system (Dionex, Amsterdam, Netherlands), coupled to a Q-TOF. Samples were loaded onto a C18 pre-column for concentration and desalting. After loading, the pre-column was placed in line for separation by a fused-silica microcapillary column (75 µm i.d. x 250 mm) packed with 5 µm C18 reversed-phase material (Dionex). Solvent A was 4 mM ammonium acetate/water. Solvent B was 2 mM ammonium acetate in 80% acetonitrile/water. Both solvents were adjusted to pH 3.0 with formic acid. A

binary gradient of 15% to 40% B within 120 min was performed, applying a flow rate of 200 µl/min reduced to approximately 300 nl/min by the Ultimate split-system. A gold coated glass capillary (PicoTip, New Objective, Cambridge, MA, USA) was used for introduction into the micro-ESI source. In MS/MS experiments, sequence information was obtained by interpretation of fragment spectra using computer-assisted database (NCBInr, non-redundant protein database) searching tools (MASCOT, Matrix Science, London, UK) [24]. In order to differentially quantify the identified peptides, peptide signals in mass chromatograms from serial LC-MS runs (runs performed directly one after the other using the same settings) were summed and quantification was done from relative peak heights in the corresponding mass spectra.

2.3.1.4 Results

Proteomic analysis of the constitutive MHC-II ligandome: Source proteins of HLA-DR presented peptides are allocated throughout the cell

We analyzed the constitutive human leukocyte antigen (HLA)-DR peptide repertoire of human Awells cells by mass spectrometry and were able to identify 404 peptides from 173 different core sequences (Table 2.3.1.1), some of them posttranslationally modified (Table 2.3.1.2). This is the largest number of MHC-presented peptides ever reported from a single experiment. In order to classify the source proteins according to their cellular localization (Figure 2.3.1.1) and function (Figure 2.3.1.2), we used the DAVID program [25] and the Gene Ontology (GO) classifications [26]. In contrast to the situation observed for MHC-I [27], the largest fraction of MHC-II source proteins, namely 41.1%, belonged to membrane proteins, which is in concordance with conventional MHC-II antigen processing via the endosomal/lysosomal pathway. However, a rather large proportion of source proteins (34.9%) is localized intracellularly – meaning the contents of a cell contained within the plasma membrane, excluding large vacuoles and secretory or ingested material (GO classifications) –, the site where MHC-I peptide processing is expected to take place. Furthermore, we could identify peptides from proteins localized in virtually every cell compartment: 10.1% lysosome, 9.2% nucleus, 4.0% cytoskeleton, 3.0% Golgi apparatus, 2.0% ER, 1.2% ribosome, 0.7% peroxisome and 0.2% mitochondrion.

Table 2.3.1.1. Sequences of peptides eluted from HLA-DR. Peptides are arranged according to their HLA-DR4 binding motive (http://www.syfpeithi.de), indicated by score and rank. Anchor amino acids are printed bold.

Gene Symbol	Peptide Sequence	Entrez Gene ID	Score	Rank
	-3 -2 -1 **1** 2 3 **4** 5 **6** 7 8 **9** +1 +2 +3			
HLA-A	**F** V R **F** D **S** D A **A** S Q R	3105	28	1/256
	F V R **F** D **S** D A **A** S Q R M E	3105		
	Q **F** V R **F** D **S** D A **A** S Q R M E	3105		
	T Q **F** V R **F** D **S** D A **A** S Q	3105		
	T Q **F** V R **F** D **S** D A **A** S Q R	3105		
	T Q **F** V R **F** D **S** D A **A** S Q R M	3105		
	T Q **F** V R **F** D **S** D A **A** S Q R M E P	3105		
	D T Q **F** V R **F** D **S** D A **A** S Q	3105		
	D T Q **F** V R **F** D **S** D A **A** S Q R	3105		
	D T Q **F** V R **F** D **S** D A **A** S Q R M	3105		
	D T Q **F** V R **F** D **S** D A **A** S Q R M E	3105		
	D T E **F** V R **F** D **S** D A **A** S Q R M E	3105		
	D T Q **F** V R **F** D **S** D A **A** S Q R M E P	3105		
	D T E **F** V R **F** D **S** D A **A** S Q R M E P	3105		
	D T E **F** V R **F** D **S** D A **A** S Q R M o E P	3105		
	D T Q **F** V R **F** D **S** D A **A** S Q R M E P R	3105		
	D T Q **F** V R **F** D **S** D A **A** S Q R M E P R A P	3105		
	D D T **Q** F V **R** F **D** S D **A** A S Q R	3105		
	D D T Q **F** V R **F** D **S** D A **A** S Q R M E	3105		
	D D T Q **F** V R **F** D **S** D A **A** S Q R M E P	3105		
	D D T Q **F** V R **F** D **S** D A **A** S Q R M E P R	3105		
	V D D T Q **F** V R **F** D **S** D A **A** S Q R	3105		
	V D D T Q **F** V R **F** D **S** D A **A** S Q R M	3105		
	V D D T Q **F** V R **F** D **S** D A **A** S Q R M E P	3105		
	V D D T Q **F** V R **F** D **S** D A **A** S Q R M E P R	3105		
	V D D T Q **F** V R **F** D **S** D A **A** S Q R M E P R A P	3105		
	V D D T Q **F** V R **F** D **S** D A **A** S Q R M E P R A P W	3105		
	V D D T Q **F** V R **F** D **S** D A **A** S Q R M E P R A P W I E	3105		
	V D D T **E** **F** V R **F** D **S** D A **A** S Q R	3105		
	T T K H K **W** E A **A** H **V** A E **Q** L R	3105	22	5/256
	K H K **W** E A **A** H **V** A E **Q** L R	3105		
HLA-B	T L **F** V R **F** D **S** D A **T** S P	3106	28	1/362
	D T L **F** V R **F** D **S** D A **T** S P R K E P R A P	3106		
	V D D T L **F** V R **F** D **S** D A **T** S P R K E P R A P	3106		
	L S S **W** T A **A** D **T** A A **Q** I T	3106		
	L S S **W** T A **A** D **T** A A **Q** I T Q	3106		
	L S S **W** T A **A** D **T** A A **Q** I T Q R	3106		
	L S S **W** T A **A** D **T** A A **Q** I T Q R K W	3106		
	L S S **W** T A **A** D **T** A A **Q** I T Q R K W E	3106		
	L S S **W** T A **A** D **T** A A **E** I T E R K W E	3106		
	D L S S **W** T A **A** D **T** A A **Q** I T	3106		
	D L S S **W** T A **A** D **T** A A **Q** I T Q	3106		
	D L S S **W** T A **A** D **T** A A **Q** I T Q R	3106		
	D L S S **W** T A **A** D **T** A A **Q** I T Q R K W	3106		
	D L S S **W** T A **A** D **T** A A **Q** I T Q R K W E	3106		
	D L S S **W** T A **A** D **T** A A **Q** I T Q R K W E A A	3106		
	D L S S **W** T A **A** D **T** A A **Q** I T Q R K W E A A R V A	3106		
	E D L S S **W** T A **A** D **T** A A **Q** I T Q R	3106		
	E D L S S **W** T A **A** D **T** A A **Q** I T	3106		
	E D L S S **W** T A **A** D **T** A A **Q** I T Q R K W	3106		
	E D L S S **W** T A **A** D **T** A A **Q** I T Q R K W E	3106		
	E D L S S **W** T A **A** D **T** A A **Q** I T Q R K W E A A R V A	3106		
	N E D L S S **W** T A **A** D **T** A A **Q** I T Q R K W	3106		
	L N E D L S S **W** T A **A** D **T** A A **Q** I T Q R K W E	3106		
	K D Y I A **L** N E **D** L **S** S W **T** A	3106	26	4/362
	G P E Y **W** D R **E** T **Q** I S **K** T N	3106	28	1/362

Table 2.3.1.1, continued

Gene Symbol	Peptide Sequence																					Entrez Gene ID	Score	Rank
	-3	-2	-1	**1**	2	3	**4**	5	**6**	7	8	**9**	+1	+2	+3									
HLA-B				**L**	R	W	**E**	P	**S**	S	Q	**S**	T	V	P	I	V	G	I	V	A	G	3106	26	4/362
HLA-C				**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P					3107	28	1/366
			T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R								3107	
			T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R				3107	
		D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R								3107	
		D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G							3107	
		D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R				3107	
		D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R	A	P		3107	
	D	D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R								3107	
	V	D	D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R			3107	
	V	D	D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R	A	P	3107	
	Y	V	D	D	T	Q	**F**	V	Q	**F**	D	**S**	D	A	**A**	S	P	R	G	E	P	R	A	P	3107	
							D	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T					3107	18	35/366
							D	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T	A				3107	
						K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T	A				3107	
						K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T					3107	
						K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W						3107	
					G	K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T					3107	
					G	K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T	A				3107	
				D	G	K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T					3107	
				D	G	K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T	A				3107	
				D	G	K	**D**	Y	I	**A**	L	**N**	E	D	**L**	R	S	W	T	A	A			3107	
	G	R	L	L	R	G	**Y**	N	Q	**F**	A	**Y**	D	G	**K**									3107	22	8/366
HLA-E				L	R	S	**W**	T	A	**V**	D	**T**	A	A	**Q**	I	S							3133	28	1/358
				L	R	S	**W**	T	A	**V**	D	**T**	A	A	**Q**	I	S	E	Q					3133	
			D	L	R	S	**W**	T	A	**V**	D	**T**	A	A	**Q**	I	S	E	Q					3133	
IGHMBP2			E	P	R	R	**Y**	G	S	**A**	A	**A**	L	P	**S**									3508	22	27/993
CLN5					G	**H**	L	K	**I**	M	**H**	D	A	**I**	G	F	R							1203	18	45/407
				L	G	**H**	L	K	**I**	M	**H**	D	A	**I**	G	F	R							1203	
HLA-DRB1							**Y**	V	R	**F**	D	**S**	D	V	**G**	E	Y							3123	22	6/266
				Q	E	E	**Y**	V	R	**F**	D	**S**	D	V	**G**	E	Y	R						3123	
			H	Q	E	E	**Y**	V	R	**F**	D	**S**	D	V	**G**	E	Y	R						3123	22	6/266
			H	Q	E	E	**Y**	V	R	**F**	D	**S**	D	V	**G**	E	Y	R	A					3123	
			H	Q	E	E	**Y**	V	R	**F**	D	**S**	D	V	**G**	E	Y	R	A	V				3123	
			G	A	G	L	**F**	I	Y	**F**	R	**N**	Q	K	**G**	H	S							3123	22	6/266
HLA-DRA			A	Q	G	A	**L**	A	N	**I**	A	**V**	D	K	**A**	N	L	E	I					3122	20	13/254
	I	Q	A	E	F	Y	**L**	N	P	**D**	Q	**S**	G	E	**F**									3122	20	13/254
HLA-DQB1				D	V	E	**V**	Y	R	**A**	V	**T**	P	L	**G**	P	P	D						3119	20	9/229
HLA-DPB1			N	R	E	E	**F**	V	R	**F**	D	**S**	D	V	**G**	E	F	R						3115	22	1/58
				R	E	E	**F**	V	R	**F**	D	**S**	D	V	**G**	E	F	R						3115	
B2M							**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y						567	22	4/119
						Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y						567	
				L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**									567	
				L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D								567	
				L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E							567	
				L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y						567	
				L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y	A					567	
			Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**									567	
			Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D								567	
			Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E							567	
			Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y						567	
			Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y	A					567	
		F	Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D									567	
		F	Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y							567	
		F	Y	L	L	Y	**Y**	T	E	**F**	T	**P**	T	E	**K**	D	E	Y	A						567	
HLA-G		V	D	D	T	Q	**F**	V	R	**F**	D	**S**	D	S	**A**	C	P	R	M	E	P				3135	28	1/338

Table 2.3.1.1, continued

Gene Symbol	Peptide Sequence	Entrez Gene ID	Score	Rank
	-3 -2 -1 **1** 2 3 **4** 5 **6** 7 8 **9** +1 +2 +3			
HLA-G	Y V D D T Q **F** V R **F** D **S** D S **A** C P R M E P R A P	3135		
HLA-DMA	F G P T **F** V S **A** V **D** G L **S** F Q	3108	22	5/257
ENO1	K E K **Y** G K **D** A **T** N V **G** D E G G	2023	22	9/433
	I K E K **Y** G K **D** A **T** N V **G** D E G G	2023		
	I K E K **Y** G K **D** A **T** N V **G** D E G	2023		
	V I K E K **Y** G K **D** A **T** N V **G** D E G G	2023		
	G V P L **Y** R H **I** A **D** L A **G** N	2023	16	66/433
	G V P L **Y** R H **I** A **D** L A **G** N S E V	2023		
	V P L **Y** R H **I** A **D** L A **G** N S E V I	2023		
	V P L **Y** R H **I** A **D** L A **G** N S E V	2023		
	V P L **Y** R H **I** A **D** L A **G** N S E	2023		
	V P L **Y** R H **I** A **D** L A **G** N	2023		
GM2A	G N **Y** R I **E** S **V** L S **S** S G	2760	22	2/193
	G N **Y** R I **E** S **V** L S **S** S G K	2760		
	T G N **Y** R I **E** S **V** L S **S** S G	2760		
	T G N **Y** R I **E** S **V** L S **S** S G K	2760		
	T G N **Y** R I **E** S **V** L S **S** S G K R	2760		
	T T G N **Y** R I **E** S **V** L S **S** S G	2760		
	T T G N **Y** R I **E** S **V** L S **S** S G K	2760		
	L G C **I** K **I** A A **S** L K G I	2760	20	6/193
	R **L** G C **I** K **I** A A **S** L K G I	2760		
SLC2A14	V P M **Y** I G **E** I **S** P T **A** L R	144195	28	1/497
MIF	S P D R V Y I **N** Y Y **D** M **N** A A **N**	4282	20	5/114
	V P D G **F** L S **E** L **T** Q Q **L** A Q	4282	28	1/114
	V P D G **F** L S **E** L **T** Q Q **L** A Q A	4282		
TFRC	C P S D **W** K T **D** S **T** C R **M** V T	7037	28	1/760
	C P S D **W** K T **D** S **T** C R **M** V T S	7037		
	C P S D **W** K T **D** S **T** C R **M** V T S E	7037		
	F T Y **I** N **L** D K **A** V L G T S N	7037	22	19/760
	Y V A **Y** S K **A** A **T** V T **G** K L	7037	22	19/760
	N S Q **L** L S **F** V R D L N Q Y R A D I	7037	26	5/760
DHX34	I R **F** V V **D** S **G** K V **K** E M	9704	22	21/576
RAD23B	L **L** Q Q **I** S **Q** H Q **E** H F	5887	20	15/409
TUBB1	A K **F** W E **V** I **S** D E **H** G I D P T	7280	22	17/444
TUBB5	E P **Y** N A **T** L **S** V H **Q** L	10382	22	17/444
	E P **Y** N A **T** L **S** V H **Q** L V E	10382		
EEF1A1	**I** E K **F** E **K** E A **A** E M G K G	1917	20	18/463
	I E K **F** E **K** E A **A** E M G K G S	1917		
	I E K **F** E **K** E A **A** E M G K G S F	1917		
	T **I** E K **F** E **K** E A **A** E M G K G S F	1917		
	S K Y **Y** V T **I** I **D** A P **G** H R D	1917	16	60/462
HSPA5	V P T K K S Q **I** F S **T** A **S** D N **Q** P T V T	3309	20	29/654
	V M R **I** I N **E** P **T** A A **A** I A Y G	3309	26	5/654
HSPA6	G E R A **M** T K **D** N **N** L L **G** R F E	3310	20	23/643
HSPA1B	R **I** I N **E** P **T** A A **A** I A	3303	26	5/641
	R **I** I N **E** P **T** A A **A** I A Y G	3303		
	V L R **I** I N **E** P **T** A A **A** I A	3303		
	V L R **I** I N **E** P **T** A A **A** I A Y	3303		
	V L R **I** I N **E** P **T** A A **A** I A Y G	3303		
	N V L R **I** I N **E** P **T** A A **A** I A	3303		
	N V L R **I** I N **E** P **T** A A **A** I A Y	3303		
	N V L R **I** I N **E** P **T** A A **A** I A Y G	3303		
HSPA8	E G E R A **M** T K **D** N **N** L L **G** K F E	3312	20	15/646
	G E R A **M** T K **D** N **N** L L **G** K F E	3312		
	G E R A **M** T K **D** N **N** L L **G** K F E L	3312		
	E R A **M** T K **D** N **N** L L **G** K F E	3312		

Table 2.3.1.1, continued

Gene Symbol	Peptide Sequence																					Entrez Gene ID	Score	Rank
				-3	-2	-1	**1**	2	3	**4**	5	**6**	7	8	**9**	+1	+2	+3						
HSPA8					E	R	A	**M**	T	K	**D**	N	**N**	L	L	**G**	K	F	E	L				3312		
						R	A	**M**	T	K	**D**	N	**N**	L	L	**G**	K	F	E					3312		
							G	**I**	L	N	**V**	S	**A**	V	D	**K**	S	T	G	K	E			3303	20	21/646
IL27RA						V	P	**Y**	R	I	**T**	V	**T**	A	V	**S**	A	S	G					9466	22	9/636
			V	G	V	P	**Y**	R	I	**T**	V	**T**	A	V	**S**	A	S	G						9466		
AHSG		I	S	R	A	Q	**F**	V	P	**L**	P	**V**	S	V	**S**	V	E							280988	22	6/359
SYNGR2		N	P	K	D	V	**L**	V	G	**A**	D	**S**	V	R	**A**	A	I	T	F					9144	26	3/224
MS4A1				S	G	P	**K**	P	L	**F**	R	**R**	M	S	**S**	L	V	G	P	T	Q	S	F	931	18	41/297
				S	G	P	**K**	P	L	**F**	R	**R**	M	S	**S**	L	V	G	P	T	Q	S		931		
					G	P	**K**	P	L	**F**	R	**R**	M	S	**S**	L	V	G	P	T	Q	S		931		
					G	P	**K**	P	L	**F**	R	**R**	M	S	**S**	L	V	G	P	T				931		
					G	P	**K**	P	L	**F**	R	**R**	M	S	**S**	L	V	G	P					931		
					G	P	**K**	P	L	**F**	R	**R**	M	S	**S**	L	V	G	P					931		
													ox											
RAB6B				L	I	P	S	**Y**	I	R	**D**	S	**T**	V	A	**V**	V	V						51560	28	1/208
RAB7				F	P	E	P	**I**	K	L	**D**	K	**N**	D	R	**A**	K	A	S	A				7879	26	2/207
CTSC					D	H	N	**F**	V	K	**A**	I	**N**	A	I	**Q**	K	S	W					1075	28	1/463
				Y	D	H	N	**F**	V	K	**A**	I	**N**	A	I	**Q**	K							1075		
				Y	D	H	N	**F**	V	K	**A**	I	**N**	A	I	**Q**	K	S						1075		
				Y	D	H	N	**F**	V	K	**A**	I	**N**	A	I	**Q**	K	S	W					1075		
				Y	D	H	N	**F**	V	K	**A**	I	**N**	A	I	**Q**	K	S	W	T				1075		
			K	Y	D	H	N	**F**	V	K	**A**	I	**N**	A	I	**Q**	K	S	W	T				1075		
				S	G	M	D	**Y**	W	I	**V**	K	**N**	S	W	**G**	T	G	W	G				1075	22	11/463
						K	V	**V**	V	Y	**L**	Q	**K**	L	D	**T**	A	Y	D					1075	20	30/463
CTSC					K	K	V	**V**	V	Y	**L**	Q	**K**	L	D	**T**	A	Y	D	D	L	G		1075		
TF								**F**	V	K	**D**	Q	**T**	V	I	**Q**	N	T	D					7018	28	1/704
					D	V	A	**F**	V	K	**D**	Q	**T**	V	I	**Q**	N	T	D					7018		
					D	V	A	**F**	V	K	**D**	Q	**T**	V	I	**Q**								7018		
				G	D	V	A	**F**	V	K	**D**	Q	**T**	V	I	**Q**								7018		
				G	D	V	A	**F**	V	K	**D**	Q	**T**	V	I	**Q**	N	T	D					7018		
CNDP2					L	A	K	**W**	V	A	**I**	Q	**S**	V	S	**A**	W	P	E					55748	28	1/475
WBSCR1				D	I	D	A	**I**	F	K	**D**	L	**S**	I	R	**S**	V	R						7458	26	1/248
GEF2			A	I	F	L	F	V	**D**	K	T	**V**	P	**Q**	S	S	**L**	T						11345	18	17/117
			A	I	F	L	F	V	**D**	K	T	**V**	P	**Q**	S	S	**L**							11345		
			A	I	F	L	F	V	**D**	K	T	**V**	P	**Q**	S	S								11345		
						F	V	**D**	K	T	**V**	P	Q	S	S	**L**								11345		
	L	P	S	E	K	A	I	F	**L**	F	V	**D**	K	T	V	P	**Q**	S	S					11345	26	2/117
	L	P	S	E	K	A	I	F	**L**	F	V	**D**	K	T	V	P	**Q**	S	S					11345		
M17S2					S	G	T	Q	**F**	V	C	**E**	T	**V**	I	R	**S**	L						4077		22/966
					S	G	T	Q	**F**	V	C	**E**	T	**V**	I	R	**S**	L	T					4077		
					S	G	T	Q	**F**	V	C	**E**	T	**V**	I	R	**S**	L	T	L	D			4077		
RAP1A						T	E	Q	**F**	T	A	**M**	R	**D**	L	Y	**M**	K	N					5906	16	30/184
CTSZ						G	T	E	**Y**	W	I	**V**	R	**N**	S	W	**G**	E	P	W				1522	22	6/303
LGMN					V	P	K	D	**Y**	T	G	**E**	D	**V**	T	P	**Q**	N						5641	22	11/433
				G	V	P	K	D	**Y**	T	G	**E**	D	**V**	T	P	**Q**	N						5641		
GAPD						L	Q	N	**I**	I	P	**A**	S	**T**	G	A	**A**	K	A	V	G			2597	26	4/334
DKFZp43400 32.1								L	**L**	Q	K	**L**	I	**L**	W	R	**V**	L							20	24/415
HIST1H2BL		V	N	D	I	F	E	R	**I**	A	S	**E**	A	**S**	R	L	**A**	H	Y	N				8340	26	2/125
		V	N	D	I	F	E	R	**I**	A	S	**E**	A	**S**	R	L	**A**							8340		
				D	I	F	E	R	**I**	A	S	**E**	A	**S**	R	L	**A**	H	Y	N				8340		
				D	I	F	E	R	**I**	A	S	**E**	A	**S**	R	L	**A**	H	Y					8340		
				D	I	F	E	R	**I**	A	S	**E**	A	**S**	R	L	**A**	H						8340		
				D	I	F	E	R	**I**	A	S	**E**	A	**S**	R	L	**A**							8340		
				D	I	F	E	R	**I**	A	S	**E**	A	**S**	R	L								8340		
APOB					S	A	S	**Y**	K	A	**D**	T	**V**	A	K	**V**	Q	G								

Table 2.3.1.1, continued

Gene Symbol	Peptide Sequence																Entrez Gene ID	Score	Rank
	-3	-2	-1	**1**	2	3	**4**	5	**6**	7	8	**9**	+1	+2	+3				
APOB			S	A	S	**Y**	K	A	**D**	T	**V**	A	K	**V**	Q	G	T				
			S	A	S	**Y**	K	A	**D**	T	**V**	A	K	**V**	Q	G	T	E			
SCAMP2		S	S	R	T	**F**	H	R	**A**	A	**S**	S	A	**A**	Q	G	A	F	10066	28	1/329
		S	S	R	T	**F**	H	R	**A**	A	**S**	S	A	**A**	Q	G	A		10066		
			S	R	T	**F**	H	R	**A**	A	**S**	S	A	**A**	Q	G	A		10066		
				R	T	**F**	H	R	**A**	A	**S**	S	A	**A**	Q	G	A	F	10066		
SCAMP3			Y	G	S	**Y**	S	T	**Q**	A	**S**	A	A	**A**	A	T			10067	22	13/347
			Y	G	S	**Y**	S	T	**Q**	A	**S**	A	A	**A**	A	T	A		10067		
			Y	G	S	**Y**	S	T	**Q**	A	**S**	A	A	**A**	A	T	A	E	10067		
DPP7						**L**	P	F	**G**	A	**Q**	S	T	**Q**	R	G	H	T	E	29952	20	23/492
IFITM1			D	R	K	**M**	V	G	**D**	V	**T**	G	A	**Q**	A	Y	A			8519	26	2/125
			D	R	K	**M**	V	G	**D**	V	**T**	G	A	**Q**	A	Y				8519		
			L	G	F	**I**	A	F	**A**	Y	**S**	V	K	**S**	R	D				8519	26	2/125
				V	P	**D**	H	V	**V**	W	**S**	L	F	**N**	T	L				8519	18	25/125
SORL1						**K**	P	G	**I**	Y	**R**	S	N	**M**	D	G	S	A	A	Y	6653	18	231/2214
	R	H	P	I	N	E	Y	**Y**	I	A	**D**	A	**S**	E	D	**Q**	V	F			6653	28	1/2214
UBE2L3	N	P	P	Y	D	K	G	A	**F**	R	I	**E**	I	**N**	F	P	**A**	E	Y	P	F	K	P	P	7332
		P	P	Y	D	K	G	A	**F**	R	I	**E**	I	**N**	F	P	**A**	E	Y	P	F	K	P	P	7332
Unnamed protein				G	P	P	**I**	G	S	**F**	T	**L**	I	D	**S**	E	V	S	Q	L		20	37/626
DNPEP							**F**	E	L	**F**	P	**S**	L	S	**H**	N	L	L	V	D	23549	22	12/475
PON2		S	P	D	D	K	**Y**	I	Y	**V**	A	**D**	I	L	**A**	H	E	I	H		5445	22	8/354
GDI2	E	P	I	E	Q	K	**F**	V	S	**I**	S	**D**	L	L	**V**	P	K				2665	22	14/445
SLC1A5		V	A	A	V	F	**I**	A	Q	**L**	S	**Q**	Q	S	**L**	D	F	V	K		6510	26	4/541
D4ST1							**L**	P	K	**Y**	I	**L**	D	F	**S**	L					113189	14	73/376
					D	V	**L**	P	K	**Y**	I	**L**	D	F	**S**	L					113189		
SIAT1						G	**I**	L	I	**V**	W	**D**	P	S	**V**	Y	H	S	D	I	P	6480	20	18/406
ABCC4					A	P	**V**	L	F	**F**	D	**R**	N	P	**I**	G	R	I	L			10257	26	19/1325
MMS19L				L	V	A	**F**	R	I	**V**	H	**D**	L	I	**S**	R	D	Y	S			64210	22	38/1030
LARGE			N	P	L	H	**F**	H	L	**I**	A	**D**	S	I	**A**	E	Q	I	L			9215	22	18/756
PPFIBP1							**M**	E	L	**P**	D	**Y**	V	L	**L**	T	A	T				8496	14	193/1005
RNASET2			S	L	E	L	**Y**	R	E	**L**	D	**L**	N	S	**V**	L	L					8635	22	3/256
ITGA4			I	D	I	S	**F**	L	L	**D**	V	**S**	S	L	**S**	R	A	E				3676	28	1/1038
			I	D	I	S	**F**	L	L	**D**	V	**S**	S	L	**S**	R	A	E	E			3676		
GNA13				L	N	I	**F**	E	T	**I**	V	**N**	N	R	**V**	F	S					10672	28	1/377
TIP120A		L	E	A	L	D	**I**	M ox	A	**D**	M	**L**	S	R	**Q**	G	G					55832	20	65/1230
		L	E	A	L	D	**I**	M	A	**D**	M	**L**	S	R	**Q**	G						55832		
ITGB7		L	F	F	F	L	**V**	E	D	**D**	A	**R**	G	T	**V**							3695	26	10/798
PGK1				R	V	V	**M**	R	V	**D**	F	**N**	V	P	**M**	K	N					5230	26	4/417
		G	P	V	G	V	**F**	E	W	**E**	A	**F**	A	R	**G**	T						5230	16	63/417
ATIC						L	**V**	E	F	**A**	R	**N**	L	T	**A**	L	G	L	N	L	V	471	26	6/592
RPS13			L	P	P	N	**W**	K	Y	**E**	S	**S**	T	A	**S**	A						6207	28	1/150
RPS10						D	**R**	D	T	**Y**	R	**R**	S	A	**V**	P	P	G	A	D		6204	12	31/165
					A	D	**R**	D	T	**Y**	R	**R**	S	A	**V**	P	P	G	A	D		6204		
							R	D	T	**Y**	R	**R**	S	A	**V**	P	P	G	A	D		6204		
CTSD							**L**	S	R	**D**	P	**D**	A	Q	**P**	G	G	E				1509	14	88/412
				I	F	S	**F**	Y	L	**S**	R	**D**	P	D	**A**	Q	P	G				1509	16	75/412
NAPB					D	Y	**Y**	K	G	**E**	E	**S**	N	S	**S**	A	N	K				63908	28	1/298
CCT2				S	L	M	**V**	T	N	**D**	G	**A**	T	I	**L**	K	N					10576	20	24/535
CPD		V	P	G	T	Y	K	**I**	T	A	**S**	A	**R**	G	Y	**N**						1362	20	72/1380
		V	P	G	T	Y	K	**I**	T	A	**S**	A	**R**	G	Y	**N**	P	V				1362		
SLAMF6			D	T	G	S	**Y**	R	A	**Q**	I	**S**	T	K	**T**	S	A	K				114836	22	6/331
KIAA1691				G	S	S	**Y**	G	S	**E**	T	**S**	I	P	**A**	A	A	H				80727	28	1/558
CPNE3				V	A	R	**F**	A	A	**A**	A	**T**	Q	Q	**Q**	T	A					8895	28	1/537
LY6E				K	P	T	**I**	C	S	**D**	Q	**D**	N	Y	**C**	V	T					4061	14	25/131

Table 2.3.1.1, continued

Gene Symbol	Peptide Sequence															Entrez Gene ID	Score	Rank
	-3	-2	-1	**1**	2	3	**4**	5	**6**	7	8	**9**	+1	+2	+3			
LY6E			L	K	P	T	**I**	C *C*	S	**D**	Q	**D**	N	Y	**C**	V	T			4061		
PSAP				G	P	S	**Y**	W	C	**Q**	N	**T**	E	T	**A**	A	Q			5660	22	8/524
C19orf10				T	E	E	**F**	E	V	**T**	K	**T**	A	V	**A**	H	R	P	G	56005	22	5/173
NEDD4L			D	G	R	T	**F**	Y	I	**D**	H	**N**	S	K	**I**	T	Q			23327	28	1/854
UBQLN1			N	P	D	T	**L**	S	A	**M**	S	**N**	P	R	**A**	M	Q			29979	20	16/589
ALDOA	A	P	G	K	G	I	**L**	A	A	**D**	E	**S**	T	G	**S**	I	A			226	26	2/363
TNFAIP3					E	I	**I**	H	K	**A**	L	**I**	D	R	**N**	I	Q			7128	20	38/790
IMPA1		Y	P	S	H	S	**F**	I	G	**E**	E	**S**	V	A	**A**	G	E	K		3612	28	1/227
VDAC2				A	A	K	**Y**	Q	L	**D**	P	**T**	A	S	**I**	S	A			7417	28	1/347
PPGB				L	P	G	**L**	A	K	**Q**	P	**S**	F	R	**Q**	Y	S	G		5476	20	30/480
COCH					R	R	**F**	N	L	**Q**	K	**N**	F	V	**G**	K	V	A		1690	16	84/550
			G	Q	R	R	**F**	N	L	**Q**	K	**N**	F	V	**G**	K	V	A		1690		
		I	G	Q	R	R	**F**	N	L	**Q**	K	**N**	F	V	**G**	K	V	A	L	1690		
TAX1BP1				H	K	G	E	**I**	R	G	**A**	S	**T**	P	F	**Q**	F	R			8887	26	2/789
SEMA7A					I	S	I	**Y**	S	S	**E**	R	**S**	V	L	**Q**					8482	28	1/666
STX6				N	P	R	K	**F**	N	L	**D**	A	**T**	E	L	**S**	I	R			10228	28	1/255
				N	P	R	K	**F**	N	L	**D**	A	**T**	E	L	**S**	I	R	K		10228		
				N	P	R	K	**F**	N	L	**D**	A	**T**	E	L	**S**	I	R	K	A	10228		
PTPRC		S	P	G	E	P	Q	**I**	I	F	**C**	R	**S**	E	A	**A**	H	Q	G		5788	20	65/1304
		S	P	G	E	P	Q	**I**	I	F	**C**	R	**S**	E	A	**A**	H	Q	G	V I	5788		
IGLC1					K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T				3537	18	9/105
					K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V			3537		
			S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T				3537		
			S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V			3537		
			S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E	K T	3537		
					K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E	K	3537		
					K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E		3537		
				H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V			3537		
				H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E		3537		
			S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E		3537		
		K	S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S**	T	V	E		3537		
	T	P	E	Q	W	K	S	H	K	S	Y	**S**	C	Q	**V**	T	**H**	E	G	**S** T V E	3537		
IGHM							G	P	T	**T**	Y	K	**V**	T	**S**	T	L	**T**	I	K	3507	18	44/454
							G	P	T	**T**	Y	K	**V**	T	**S**	T	L	**T**	I	K E	3507	18	44/454
					S	G	P	T	**T**	Y	K	**V**	T	**S**	T	L	**T**	I	K		3507		
					S	G	P	T	**T**	Y	K	**V**	T	**S**	T	L	**T**	I	K	E S D W L	3507		
				E	S	G	P	T	**T**	Y	K	**V**	T	**S**	T	L	**T**	I	K	E S D W L	3507		
IGH@						Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**				3492	26	1/33
				T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D					3492		
				T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**				3492		
				T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**	A			3492		
			N	T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**				3492		
			N	T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**	A			3492		
		K	N	T	L	Y	L	Q	**M**	N	S	**L**	K	**T**	E	D	**T**	A			3492		
UBA52				S	D	Y	N	**I**	Q	K	**E**	S	**T**	L	H	**L**	V				7311	26	1/76
				D	Y	N	**I**	Q	K	**E**	S	**T**	L	H	**L**	V	L	R		7311		
ACLY			Y	P	E	E	A	**Y**	I	A	**D**	L	**D**	A	K	**S**	G	A	S		47	22	24/1001
HTGN29					R	G	**Y**	M	E	**I**	E	**Q**	S	V	**K**	S	F	K			56951	28	1/265
WDR1				A	P	S	G	**F**	Y	I	**A**	S	**G**	D	V	**S**	G	K	L	R	9948	22	12/606
				A	P	S	G	**F**	Y	I	**A**	S	**G**	D	V	**S**	G	K	L		9948		
ATP1A1					I	V	V	**Y**	T	G	**D**	R	**T**	V	M	**G**	R	I	A	T	476	22	31/1023
					I	V	V	**Y**	T	G	**D**	R	**T**	V	M	**G**	R	I	A		476		
CTSS					G	K	E	**Y**	W	L	**V**	K	**N**	S	W	**G**	H	N			1520	22	6/331
				T	T	A	F	**Q**	Y	I	**I**	D	**N**	K	G	**I**	D				1520	18	40/331
				T	T	A	F	**Q**	Y	I	**I**	D	**N**	K	G	**I**	D	S	D		1520		

Table 2.3.1.1, continued

Gene Symbol	Peptide Sequence																Entrez Gene ID	Score	Rank
	-3	-2	-1	**1**	2	3	**4**	5	**6**	7	8	**9**	+1	+2	+3				
CTSS	K	N	L	K	F	V	M	L	H	**N**	L	E	**H**	S	M		1520	22	6/331
CLTCL1		L	E	K	**I**	V	L	**D**	N	**S**	V	F	**S**	E	H	R	N	8218	26	17/1640
RNF13	I	P	S	V	**F**	I	G	**E**	S	**S**	A	N	**S**	L	K	D		11342	28	1/381
MTP18	I	P	I	I	**I**	H	P	**I**	D	**R**	S	V	**D**					51537	20	9/206
LAPTM5		L	P	S	**Y**	E	E	**A**	L	**S**	L	P	**S**	K	T	P		7805	28	1/262
		L	P	S	**Y**	E	E	**A**	L	**S**	L	P	**S**	K	T	P	E	7805		
		L	P	S	**Y**	E	E	**A**	L	**S**	L	P	**S**	K	T	P	E G	7805		
				G	**Y**	L	R	**I**	A	**D**	L	I	**S**	S	F			7805	22	12/262
	V	V	L	P	S	**Y**	E	E	**A**	L	**S**	L	P	**S**	K	T	P E	7805	28	1/262
TPI1					**L**	K	P	**E**	F	**V**	D	I	**I**	N	A	K	Q	7167	20	10/248
YWHAE		R	A	S	**W**	R	I	**I**	S	**S**	I	E	**Q**	K	E	E		7531	28	1/255
GLG1		K	V	N	**L**	L	K	**I**	K	**T**	E	L	**C**	K	K	E	V	2734	20	36/1179
			V	N	**L**	L	K	**I**	K	**T**	E	L	**C**	K	K	E	V	2734		
	D	P	E	L	D	Y	T	**L**	M	R	**V**	C	**K**	Q	M	**I**	K	2734	20	36/1179
		L	G	K	**W**	C	S	**E**	K	**T**	E	T	**G**	Q	E			2734	22	20/1179
PPIA	V	S	F	E	**L**	F	A	**D**	K	**V**	P	K	**T**	A	E	N		5478	20	6/164
CCR7					**I**	A	Y	**D**	V	**T**	Y	S	**L**	A	C	V	R	1236	26	6/378
				N	**I**	A	Y	**D**	V	**T**	Y	S	**L**	A	C	V	R	1236		
PTGFRN		V	P	G	**F**	A	D	**D**	P	**T**	E	L	**A**	C	R	V		5738	28	1/879
SLC3A2		T	G	A	**L**	Y	R	**I**	G	**D**	L	Q	**A**	F	Q	G	H G	6520	20	26/529
ALB		S	P	D	**L**	P	K	**L**	K	**P**	D	P	**N**	T	L	C	D E F	280717	20	30/607
RAB4A		G	A	L	**L**	V	Y	**D**	I	**T**	S	R	**E**	T	Y	N		5867	20	19/213
CCT7		A	T	Q	**Y**	F	A	**D**	R	**D**	M	F	**C**	A	G	R	V P	10574	16	106/543
	V	A	T	Q	**Y**	F	A	**D**	R	**D**	M	F	**C**	A	G	R	V P	10574		
GPC4	V	T	R	A	**F**	V	A	**A**	R	**T**	F	A	**Q**	G	L			2239	28	1/556
MAP1LC3B			T	P	**I**	S	E	**V**	Y	**E**	S	E	**K**	D	E	D	G F L	81631	20	11/124
			T	P	**I**	S	E	**V**	Y	**E**	S	E	**K**	D	E	D	G F L Y	81631		
TNFSF9		G	P	L	S	**W**	Y	S	**D**	P	**G**	L	A	**G**	V	S		8744	16	84/254
VCP	Q	L	I	Y	I	P	**L**	P	D	**E**	K	**S**	R	V	**A**			7415	26	5/806
MAN2B1						**V**	D	Y	**F**	L	**N**	V	A	**T**	A	Q	G R Y Y	4125	26	7/1010
		H	P	P	E	**L**	L	F	**S**	A	**S**	L	P	**A**	L	G		4125	20	68/1010
		H	P	P	E	**L**	L	F	**S**	A	**S**	L	P	**A**	L	G	F S	4125		
		H	P	P	E	**L**	L	F	**S**	A	**S**	L	P	**A**	L	G	F S T	4125		
CLSTN3		N	P	P	L	**F**	A	L	**D**	K	**D**	A	P	**L**	R	Y		9746	22	21/956
Dlc2			M	E	K	**Y**	N	I	**E**	K	**D**	I	A	**A**	Y	I	K	140735	22	3/89
LNPEP	D	V	R	K	L	**Y**	W	L	**M**	K	**S**	S	L	**N**	G	D	N	4012	22	28/1025
ANXA2			V	P	K	**W**	I	S	**I**	M	**T**	E	R	**S**	V	P	H	302		28/338
		D	V	P	K	**W**	I	S	**I**	M	**T**	E	R	**S**	V	P	H L	302		
		D	V	P	K	**W**	I	S	**I**	M	**T**	E	R	**S**	V	P	H L Q	302		
C10orf128	T	G	K	T	P	G	A	E	I	D	**F**	K	Y	**A**	L	**I**	G T	**A**	V G V A	170371	22	3/155
C6orf211							**I**	P	W	**F**	V	**S**	D	T	**T**	I	H D F N	79624	26	4/441
IL6ST			I	E	V	**W**	V	E	**A**	E	**N**	A	L	**G**	K	V	T	3572	22	24/918
CD74		M	H	H	W	**L**	L	F	**E**	M	**S**	R	H	**S**	L	E		972	26	2/296
		A	T	P	L	**L**	M	Q	**A**	L	**P**	M	G	**A**	L	P	Q G P	972	20	14/296
DDX1				G	**Y**	L	P	**N**	Q	**L**	F	R	**T**	F				1653		
CREG					**W**	G	A	**L**	A	**T**	I	S	**T**	L	E	A	V R	8804	28	1/220
VPS35	D	P	D	P	E	D	**F**	A	D	**E**	Q	**S**	L	V	**G**	R	F I	55737	22	31/796
HPCL2		A	I	P	F	V	**I**	E	K	**A**	V	**R**	S	S	**I**	Y		26061	26	2/578
		A	I	P	F	V	**I**	E	K	**A**	V	**R**	S	S	**I**	Y	G			
ACAA1		L	K	P	A	**F**	K	K	**D**	G	**S**	T	T	**A**	G	N		30	28	1/424
KIAA0494						**F**	S	Q	**F**	L	**G**	D	P	**V**	E	K	A A Q	9813	22	13/495
APOD			Q	E	**L**	R	A	**D**	G	**T**	V	N	**Q**	I	E	G				
CD38		R	D	M	**F**	T	L	**E**	D	**T**	L	L	**G**	Y	L	A	D	952	22	7/300
		R	D	M	**F**	T	L	**E**	D	**T**	L	L	**G**					952		
		R	D	M	**F**	T	L	**E**	D	**T**	L							952		
	V	Q	R	D	M	**F**	T	L	**E**	D	**T**	L						952		

Table 2.3.1.1, continued

Gene Symbol	Peptide Sequence	Entrez Gene ID	Score	Rank
	-3 -2 -1 **1** 2 3 **4** 5 **6** 7 8 **9** +1 +2 +3			
ACTG1	W I S K **Q** E Y **D** E **S** G P **S** I V H R K C F	71	18	40/375
	T D Y **L** M K **I** L **T** E R **G** Y S	71	20	20/375
	T D Y **L** M K **I** L **T** E R **G** Y S F	71		
	T D Y **L** M K **I** L **T** E R **G** Y S F T	71		
	R D L T D Y **L** M K **I** L **T** E R **G** Y S	71		
	G R D L T D Y **L** M K **I** L **T** E R **G** Y S	71		
RAB8	A **F** F T **L** A **R** D I **K** A K M D	4218	28	1/207
	N A **F** F T **L** A **R** D I **K** A K M D	4218		
AGRN	G R S **F** L A **F** P **T** L R **A** Y H T	375790	28	1/2026
	G R S **F** L A **F** P **T** L R **A** Y H T L	375790		
	E G R S **F** L A **F** P **T** L R **A** Y H T L	375790		
	P V P A F E G R S **F** L A **F** P **T** L R **A** Y H T L	375790		
	A P V P A F E G R S **F** L A **F** P **T** L R **A** Y H T L	375790		
IGF2R	L I T **F** L C **D** R **D** A G **V** G F P E	3482	22	43/2491
	L I T **F** L C **D** R **D** A G **V** G F P	3482		
UBE2L3	K G A **F** R I **E** I **N** F P **A** E Y P F K P P	7332	28	1/154
	D K G A **F** R I **E** I **N** F P **A** E Y P F K P P	7332		

Table 2.3.1.2. Sequences of posttranslationally modified peptides eluted from HLA-DR. Peptides are arranged according to their HLA-DR4 binding motive (www.syfpeithi.de), indicated by score and rank. Anchor amino acids are printed in bold. Modifications are printed in italics: *E*, deamidation; *C*, cysteinylation; *Gl*, glycosylation.

Gene Symbol	Peptide Sequence	Entrez Gene ID	Score	Rank
	-3 -2 -1 **1** 2 3 **4** 5 **6** 7 8 **9** +1 +2 +3			
HLA-A	D T *E* **F** V R **F** D **S** D A **A** S Q R M E	3105	28	1/256
	D T *E* **F** V R **F** D **S** D A **A** S Q R M E P	3105		
	D T *E* **F** V R **F** D **S** D A **A** S Q R M E P ox	3105		
LY6E	L K P T **I** *C* S **D** Q **D** N Y **C** V T *C*	4061	14	25/131
CD53 [23]	I H R **Y** H S **D** *N* **S** T K **A** A W D *Gl*		28	1
	S I H R **Y** H S **D** *N* **S** T K **A** A W D *Gl*			

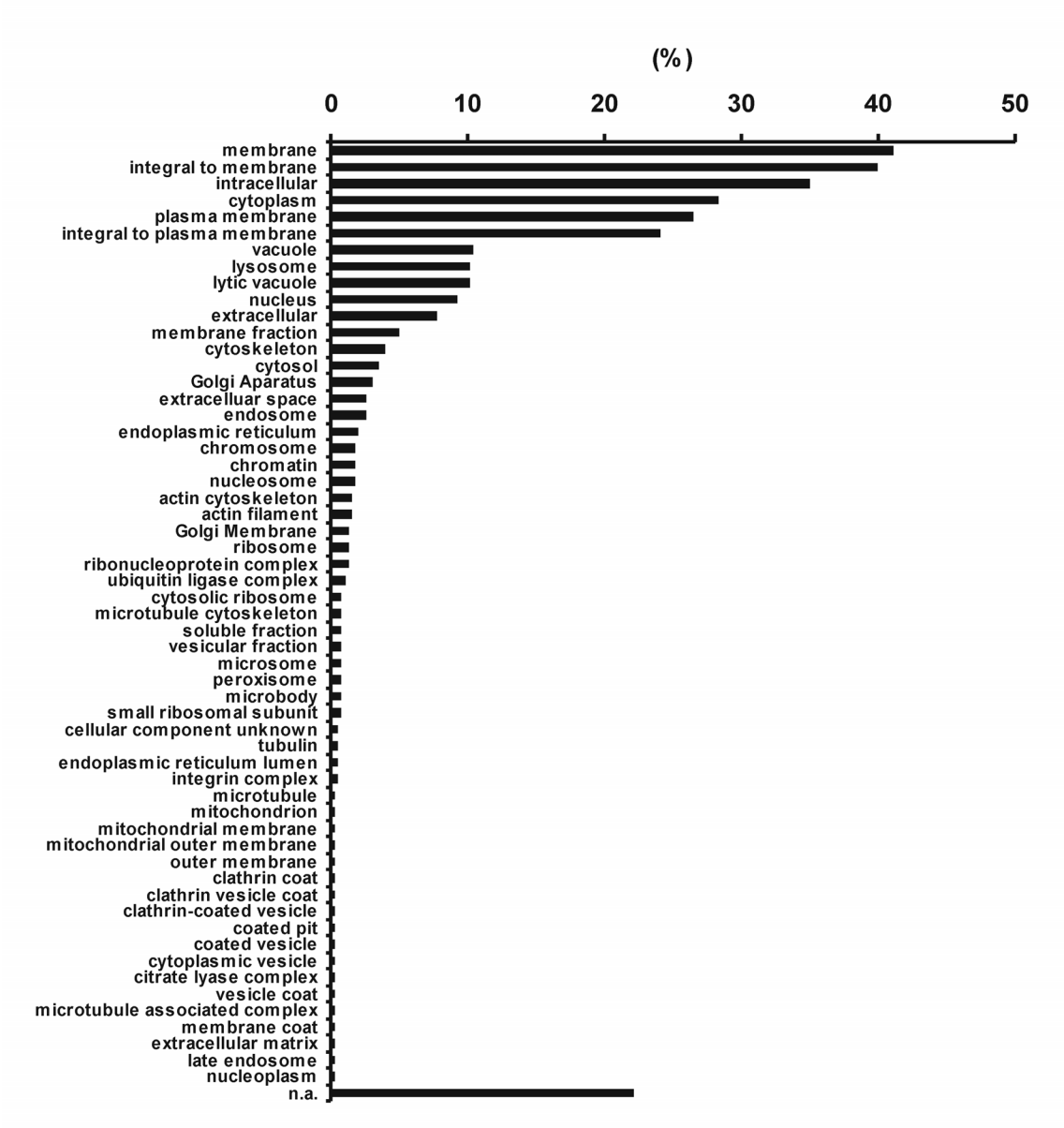


Figure 2.3.1.1. Cellular distribution of source proteins of HLA-DR ligands. Peptides were isolated from 5.7×10^{10} cells, separated via HPLC, and subsequently analyzed by nanoflow ESI MS/MS. Displayed are percentages of peptides falling in each GO category of source proteins. The 404 identified peptides represent 100%. As some of the source proteins could be found in more than one compartment, the total is higher than 100%.

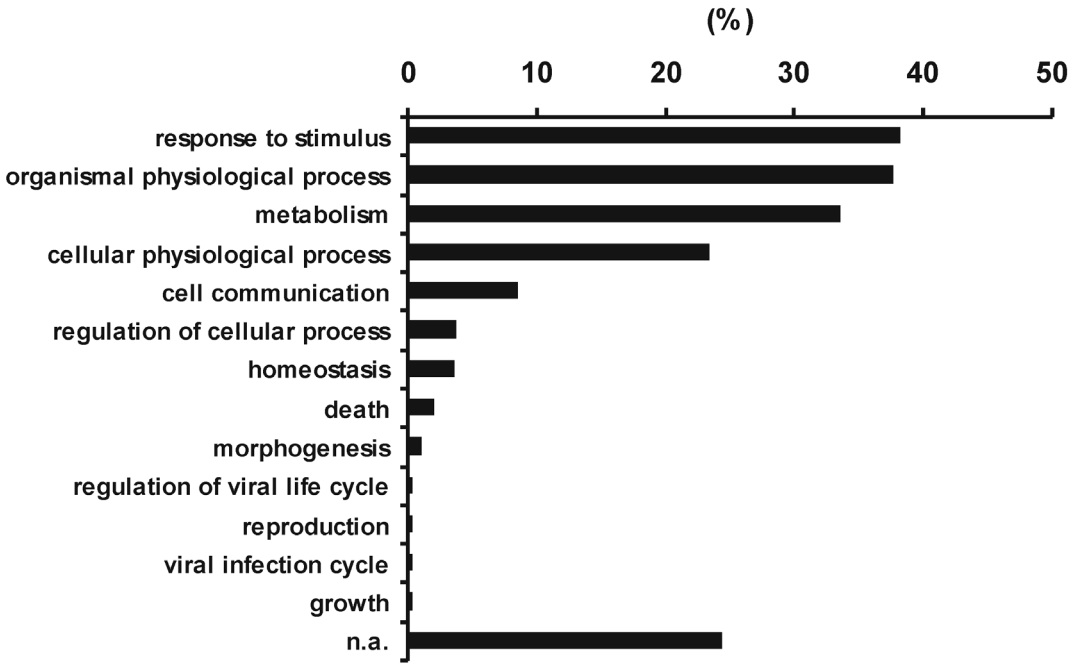


Figure 2.3.1.2. Biological processes in which HLA class II peptide source proteins take part. The major part of proteins was involved in responses to stimuli and in organismal physiological processes. Displayed are percentages of peptides falling in each GO category of source proteins (404 identified peptides represent 100%). As some of the source proteins could be found in more than one compartment, the total is higher than 100%.

Induction of autophagy by starvation

Next, we induced macroautophagy in Awells cells by deprivation of serum and amino acids [15, 28] in order to perform a comparative, quantitative HLA-DR ligandome analysis between cells undergoing autophagy and control cells by mass spectrometry. After 6 h and 24 h starvation, an increase in size and total number of autophagic vacuoles became evident when compared to non-starved control cells (Figure 2.3.1.3A-C). After 6 h starvation, the formation of autophagic vacuoles, assessed by the overall incorporation of monodansylcadaverine (MDC), had already reached the maximum and could not be increased any further by 24 h starvation (Figure 2.3.1.3D). We were able to inhibit MDC incorporation by 3-methyladenine, a specific inhibitor of autophagy (data not shown). We would like to point out that we detected basal levels of autophagy in cells even if they were not kept in starvation medium. This indicates that Awells cells already display a constitutive level of autophagy, which can be considerably enhanced by starvation. This has already been demonstrated for other cell lines [15, 28].

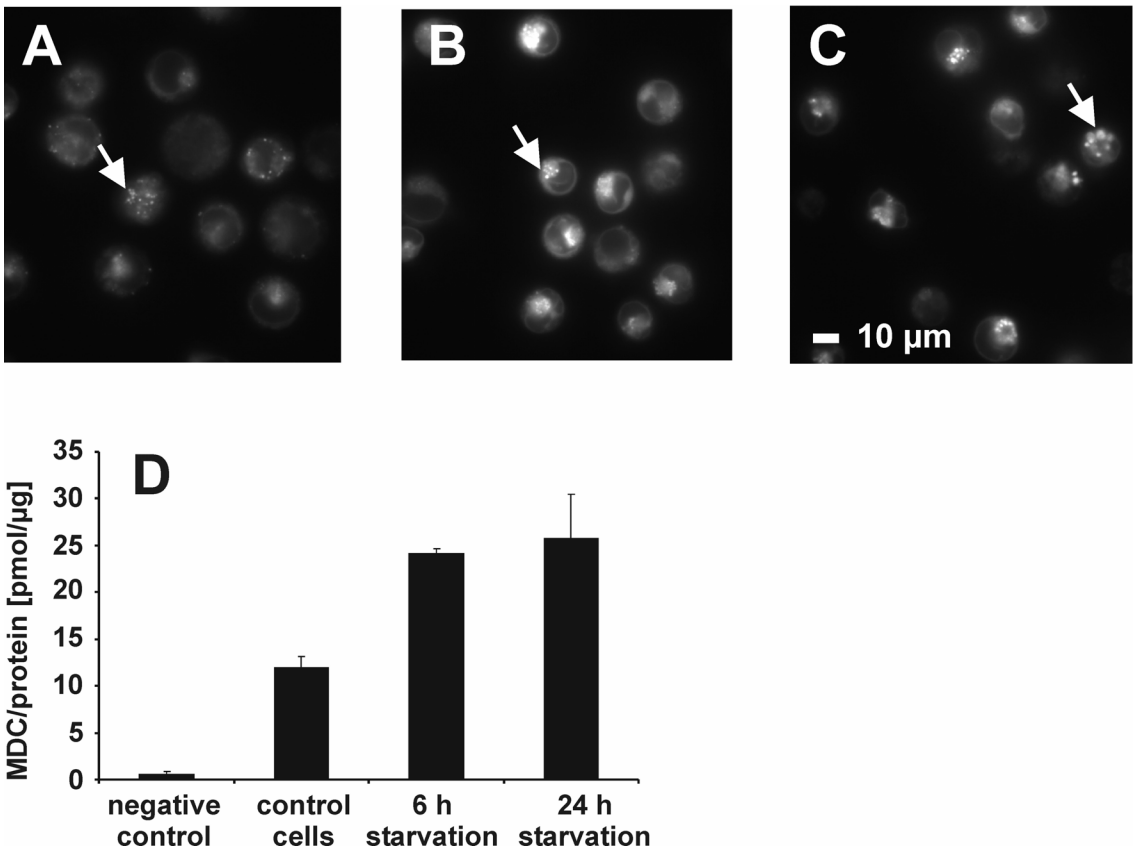


Figure 2.3.1.3. Starvation enhances the level of autophagic vacuoles. Autophagic vacuoles were stained with the specific dye monodansylcadaverine (MDC) [14] and analyzed by fluorescence microscopy or fluorescence spectroscopy. Awells cells were incubated for **(A)** 24 h in DMEM (control cells), **(B)** 6 h HBSS or **(C)** 24 h HBSS (starved cells), subsequently for 10 min with monodansylcadaverine (MDC), washed and immediately analyzed by fluorescence microscopy. Autophagic vacuoles are marked with an arrow. **(D)** Intracellular MDC measurement by fluorescence spectroscopy, unstained cells were used as negative control.

In order to obtain a detailed impression of human genes involved in autophagy, a comparative gene expression analysis using oligonucleotide microarrays was performed. Genes that were reproducibly up- or downregulated at 6 h and 24 h were assigned to functional categories based on the Gene Ontology (GO) classification system [26]. Categories showing a significantly enhanced proportion of regulated genes compared to the overall proportion of regulated genes were filtered out and are shown in Table 2.3.1.3 and Table 2.3.1.4. Several characteristics of autophagy as a process to ensure cell survival in a nutritionally deprived environment are reflected in these categories, exhibiting a distinct transcriptional signature of starved cells.

Table 2.3.1.3. Gene ontology (GO) categories significantly upregulated under starvation. Genes fulfilling the up-regulation requirements are shown (2 means 200% of mRNA was detected in starved compared with control cells). The first column indicates the GO category together with the GO ID and the GO system (BP, biological process; CC, cellular component; MF, molecular function).

Gene category (GO ID, GO system)	Entrez Gene ID	Gene symbol	Gene title	x-fold over-expression	
				6 h	24 h
Vesicular transport and membrane trafficking					
ARF guanyl-nucleotide exchange factor activity (5086, MF)	26269	FBXO8	F-box only protein 8	2.1	4.0
	9267	PSCD1	Pleckstrin homology, Sec7 and coiled-coil domains 1	2.1	2.5
	9265	PSCD3	Pleckstrin homology, Sec7 and coiled-coil domains 3	2.6	2.6
Small GTPase mediated signal transduction (7264, BP)	399	ARHH	Ras homolog gene family, member H	1.4	2.1
	23433	ARHQ	Ras homolog gene family, member Q	2.6	3.7
	221079	ARL8	ADP-ribosylation factor-like 8	2.5	4.3
	55207	FLJ10702	Hypothetical protein FLJ10702	2.1	3.0
	2669	GEM	GTP binding protein overexpressed in skeletal muscle	3.5	8.0
	2889	GRF2	Guanine nucleotide-releasing factor 2	1.6	1.4
	3845	KRAS2	v-Ki-ras2 Kirsten rat sarcoma 2 viral oncogene homolog	1.5	2.6
	51762	LOC51762	RAB-8b protein	1.7	2.0
	51735	PDZGEF2	PDZ domain containing guanine nucleotide exchange factor (GEF) 2	4.0	4.0
	5862	RAB2	RAB2, member RAS oncogene family	1.6	2.5
	84932	RAB2B	RAB2B, member RAS oncogene family	1.4	2.1
	5868	RAB5A	RAB5A, member RAS oncogene family	1.5	2.0
	9367	RAB9A	RAB9A, member RAS oncogene family	2.3	2.3
	10890	RAB10	RAB10, member RAS oncogene family	1.4	2.3
	51552	RAB14	RAB14, member RAS oncogene family	1.4	1.7
	23011	RAB21	RAB21, member RAS oncogene family	1.4	2.5
	57403	RAB22A	RAB22A, member RAS oncogene family	1.6	2.1
	5898	RALA	v-Ral simian leukemia viral oncogene homolog A	1.4	2.3
	57826	RAP2C	RAP2C, member of RAS oncogene family	1.6	2.1
	6009	RHEB	Ras homolog enriched in brain	1.6	1.5
	121268	RHEBL1	Ras homolog enriched in brain like 1	5.3	3.0
	6016	RIT1	Ras-like without CAAX 1	1.5	2.3
	64121	RRAGC	Ras-related GTP binding C	2.6	2.8
	22800	RRAS2	Related RAS viral (r-ras) oncogene homolog 2	1.5	1.9
	8036	SHOC2	Soc-2 suppressor of clear homolog (*C. elegans*)	1.7	2.1
	6478	SIAH2	Seven in absentia homolog 2 (*Drosophila*)	2.1	3.0
	7248	TSC1	Tuberous sclerosis 1	1.9	2.1
Amino acid transport and metabolism					
Amino acid transport (6865, BP)	6558	SLC12A2	Solute carrier family 12 (sodium/potassium/chloride transporters), member 2	1.4	3.0
Neutral amino acid transporter activity (15175, MF)	6509	SLC1A4	Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	2.1	4.0
	6510	SLC1A5	Solute carrier family 1 (neutral amino acid transporter), member 5	1.4	3.0
Amino acid permease activity (15359, MF)	81539	SLC38A1	Solute carrier family 38, member 1	2.0	3.7
	54407	SLC38A2	Solute carrier family 38, member 2	4.3	4.9
	6520	SLC3A2	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	2.6	4.6
	6541	SLC7A1	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	2.8	3.7
	23657	SLC7A11	Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	3.2	4.6
	8140	SLC7A5	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	3.0	4.9
Serine family amino acid biosynthesis (9070, BP)	1491	CTH	Cystathionase (cystathionine gamma-lyase)	5.7	18.4
	26227	PHGDH	Phosphoglycerate dehydrogenase	2.6	6.1
	29968	PSAT1	Phosphoserine aminotransferase 1	3.7	9.8
	5723	PSPH	Phosphoserine phosphatase	2.3	6.1

Effects on cell cycle					
Cell cycle arrest (7050, BP)	1026	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.9	2.6
	1027	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.5	3.2
	1647	GADD45A	Growth arrest and DNA-damage-inducible, alpha	2.8	3.5
	8522	GAS7	Growth arrest-specific 7	1.6	3.0
	283431	LOC283431	Hypothetical protein LOC283431	1.6	4.3
	5325	PLAGL1	Pleiomorphic adenoma gene-like 1	1.4	2.0
	23645	PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	8.0	5.7
	83667	SESN2	Sestrin 2	4.3	12.1
Protein phosphatase type 2C activity (15071, MF)	5494	PPM1A	Protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform	2.3	2.6
	8493	PPM1D	Protein phosphatase 1D magnesium-dependent, delta isoform	2.1	4.6
Apoptosis					
Apoptosis (6915, BP)	64651	AXUD1	AXIN1 up-regulated 1	2.6	4.6
	596	BCL2	B-cell CLL/lymphoma 2	1.6	4.9
	597	BCL2A1	BCL2-related protein A1	1.9	1.9
	598	BCL2L1	BCL2-like 1	1.4	1.9
	329	BIRC2	Baculoviral IAP repeat-containing 2	1.6	2.8
	331	BIRC4	Baculoviral IAP repeat-containing 4	1.5	1.5
	664	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	1.4	1.7
	665	BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	2.1	2.6
	8837	CFLAR	CASP8 and FADD-like apoptosis regulator	1.7	1.5
	8087	FXR1	Fragile X mental retardation, autosomal homolog 1	1.5	1.6
	1647	GADD45A	Growth arrest and DNA-damage-inducible, alpha	2.8	3.5
	4616	GADD45B	Growth arrest and DNA-damage-inducible, beta	2.6	4.6
	8870	IER3	Immediate early response 3	1.4	1.5
	51747	LUC7A	Cisplatin resistance-associated overexpressed protein	1.4	2.1
	4170	MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	2.1	1.7
	4790	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	1.6	2.1
	7262	PHLDA2	Pleckstrin homology-like domain, family A, member 2	3.0	2.6
	23645	PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	8.0	5.7
	5885	RAD21	RAD21 homolog (*S. pombe*)	1.5	2.5
	8767	RIPK2	Receptor-interacting serine-threonine kinase 2	1.7	3.0
	64121	RRAGC	Ras-related GTP binding C	2.6	2.8
	6446	SGK	Serum/glucocorticoid regulated kinase	9.2	1.7
	23411	SIRT1	Sirtuin 1 (*S. cerevisiae*)	3.2	4.3
	9263	STK17A	Serine/threonine kinase 17a (apoptosis-inducing)	1.9	2.5
	9262	STK17B	Serine/threonine kinase 17b (apoptosis-inducing)	1.6	1.9
	7009	TEGT	Testis enhanced gene transcript (BAX inhibitor 1)	1.5	2.5
	7124	TNF	Tumor necrosis factor (TNF superfamily, member 2)	2.0	1.4
	7128	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	2.1	2.6
	8795	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b	2.5	3.0
	8793	TNFRSF10D	Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	9.2	13.9
	958	TNFRSF5	Tumor necrosis factor receptor superfamily, member 5	1.9	1.4
	355	TNFRSF6	Tumor necrosis factor receptor superfamily, member 6	1.9	2.1
	3604	TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	2.1	2.1
	970	TNFSF7	Tumor necrosis factor (ligand) superfamily, member 7	1.6	2.0
	8626	TP73L	Tumor protein p73-like	1.4	2.5
	7187	TRAF3	TNF receptor-associated factor 3	1.6	1.6
	8565	YARS	Tyrosyl-tRNA synthetase	2.1	1.7
Anti-apoptosis (6916, BP) *Apoptosis inhibitor activity* (8189, MF)	596	BCL2	B-cell CLL/lymphoma 2	1.6	4.9
	597	BCL2A1	BCL2-related protein A1	1.9	1.9
	598	BCL2L1	BCL2-like 1	1.4	1.9
	329	BIRC2	Baculoviral IAP repeat-containing 2	1.6	2.8
	331	BIRC4	Baculoviral IAP repeat-containing 4	1.5	1.5
	664	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	1.4	1.7
	673	BRAF	v-Raf murine sarcoma viral oncogene homolog B1	1.4	3.7
	8837	CFLAR	CASP8 and FADD-like apoptosis regulator	1.7	1.5
	25816	GG2-1	TNF-induced protein	1.9	2.6
	8870	IER3	Immediate early response 3	1.4	1.5
	4790	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	1.6	2.1
	5663	PSEN1	Presenilin 1 (Alzheimer disease 3)	1.7	2.8
	5055	SERPINB2	Serine (or cysteine) proteinase inhibitor, clade B, member 2	1.9	3.7
	8887	TAX1BP1	Tax1 binding protein 1	1.4	2.0
	7124	TNF	Tumor necrosis factor (TNF superfamily, member 2)	2.0	1.4

	7128	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	2.1	2.6
	355	TNFRSF6	Tumor necrosis factor receptor superfamily, member 6	1.9	2.1
Sphingoid and ceramide metabolism					
Sphingoid metabolism (46519, BP)	427	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	1.4	2.6
	8439	NSMAF	Neutral sphingomyelinase (N-SMase) activation associated factor	2.5	1.7
Ceramide metabolism (6672, BP)	5515	PPP2CA	Protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	1.4	1.5
	7357	UGCG	UDP-glucose ceramide glucosyltransferase	3.2	3.2
Regulation of transcription					
Transcription corepressor activity (3714, MF)	467	ATF3	Activating transcription factor 3	6.5	8.0
	22809	ATF5	Activating transcription factor 5	1.4	1.5
	1810	DR1	Down-regulator of transcription 1, TBP-binding	1.4	2.3
	3726	JUNB	Jun B proto-oncogene	1.7	2.0
	11278	KLF12	Kruppel-like factor 12	1.9	2.5
	4601	MXI1	MAX interacting protein 1	1.9	2.5
	23522	MYST4	MYST histone acetyltransferase (monocytic leukemia) 4	1.9	1.7
	8554	PIAS1	Protein inhibitor of activated STAT, 1	1.7	1.4
	5971	RELB	v-Rel reticuloendotheliosis viral oncogene homolog B	1.5	1.4
	6478	SIAH2	Seven in absentia homolog 2 (*Drosophila*)	2.1	3.0
	22797	TFEC	Transcription factor EC	1.4	5.3
	7528	YY1	YY1 transcription factor	2.0	2.5
Histone acetyltransferase activity (4402, MF)	1387	CREBBP	CREB binding protein (Rubinstein-Taybi syndrome)	1.6	1.9
	2033	EP300	E1A binding protein p300	1.6	2.0
	23522	MYST4	MYST histone acetyltransferase (monocytic leukemia) 4	1.9	1.7
	8202	NCOA3	Nuclear receptor coactivator 3	1.7	2.0
Nuclear membrane					
Nuclear inner membrane (5637, CC)	4000	LMNA	Lamin A/C	3.5	2.3
	23592	MAN1	Integral inner nuclear membrane protein	1.6	2.1
	5663	PSEN1	Presenilin 1 (Alzheimer disease 3)	1.7	2.8

Table 2.3.1.4. Gene ontology (GO) categories significantly downregulated under starvation. Genes fulfilling the down-regulation requirements are shown (-2 means 50% of mRNA was detected in starved compared with control cells). The first column indicates the GO category together with the GO ID and the GO system (BP, biological process; CC, cellular component; MF, molecular function).

Gene category **(GO ID, GO system)**	**Entrez gene ID**	**Gene symbol**	**Gene title**	**x-fold over-expression**	
				6 h	**24 h**
Vesicular transport					
Golgi stack (5795, CC)	23062	GGA2	Golgi associated, gamma adaptin ear containing, ARF binding protein 2	-1.5	-1.7
	23163	GGA3	Golgi associated, gamma adaptin ear containing, ARF binding protein 3	-1.6	-3.2
	2778	GNAS	GNAS complex locus	-1.5	-1.9
	4952	OCRL	Oculocerebrorenal syndrome of Lowe	-1.5	-2.3
	10040	TOM1L1	Target of myb1-like 1 (chicken)	-1.9	-1.6
DNA replication and repair					
DNA replication origin binding (3688, MF)	4999	ORC2L	Origin recognition complex, subunit 2-like (yeast)	-1.6	-1.6
	23595	ORC3L	Origin recognition complex, subunit 3-like (yeast)	-1.6	-1.5
	5001	ORC5L	Origin recognition complex, subunit 5-like (yeast)	-1.5	-2.0
Damaged DNA binding (3684, MF)	672	BRCA1	Breast cancer 1, early onset	-1.5	-2.3
	2237	FEN1	Flap structure-specific endonuclease 1	-1.6	-2.6
	2967	GTF2H3	General transcription factor IIH, polypeptide 3, 34kDa	-1.4	-1.6

	7965	JTV1	JTV1 gene	-1.5	-4.0
	5383	PMS2L5	Postmeiotic segregation increased 2-like 5	-1.7	-4.3
	5889	RAD51C	RAD51 homolog C (*S. cerevisiae*)	-1.7	-1.5
	5892	RAD51L3	RAD51-like 3 (*S. cerevisiae*)	-1.6	-1.7
	7508	XPC	Xeroderma pigmentosum, complementation group C	-1.4	-1.4
	7517	XRCC3	X-ray repair complementing defective repair in Chinese hamster cells 3	-1.4	-1.6
Ribosome formation					
Ribosome biogenesis and assembly	10969	EBNA1BP2	EBNA1 binding protein 2	-1.6	-2.5
(42254, BP)	23212	RRS1	Ribosome biogenesis regulator homolog (*S. cerevisiae*)	-2.5	-3.0
Nucleolus	1663	DDX11	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, *S. cerevisiae*)	-1.6	-2.6
(5730, CC)	54606	DDX56	DEAD (Asp-Glu-Ala-Asp) box polypeptide 56	-1.4	-4.0
	50628	GEMIN4	Gem (nuclear organelle) associated protein 4	-3.2	-10.6
	23567	JAZ	Double-stranded RNA-binding zinc finger protein JAZ	-1.4	-1.9
	84365	MKI67IP	MKI67 (FHA domain) interacting nucleolar phosphoprotein	-1.7	-1.9
	10514	MYBBP1A	MYB binding protein (P160) 1a	-1.9	-3.5
	4809	NHP2L1	NHP2 non-histone chromosome protein 2-like 1 (*S. cerevisiae*)	-1.4	-1.6
	10528	NOL5A	Nucleolar protein 5A (56kDa with KKE/D repeat)	-1.4	-1.7
	9221	NOLC1	Nucleolar and coiled-body phosphoprotein 1	-1.4	-1.4
	54512	RRP41	Exosome complex exonuclease RRP41	-2.5	-18.4
	6949	TCOF1	Treacher Collins-Franceschetti syndrome 1	-1.6	-1.7
	7343	UBTF	Upstream binding transcription factor, RNA polymerase I	-2.5	-4.3
RNA synthesis and processing					
RNA cap binding	1973	EIF4A1	Eukaryotic translation initiation factor 4A, isoform 1	-1.7	-1.7
(339, MF)	9470	EIF4EL3	Eukaryotic translation initiation factor 4E-like 3	-4.3	-5.7
	4686	NCBP1	Nuclear cap binding protein subunit 1, 80kDa	-1.5	-1.7
	22916	NCBP2	Nuclear cap binding protein subunit 2, 20kDa	-1.4	-1.7
	10073	RNUT1	RNA, U transporter 1	-1.4	-2.3
RNA elongation	5438	POLR2I	Polymerase (RNA) II (DNA directed) polypeptide I	-1.5	-2.8
(6354, BP)	51728	POLR3K	Polymerase (RNA) III (DNA directed) polypeptide K	-1.6	-2.5
	30834	ZNRD1	Zinc ribbon domain containing, 1	-1.6	-2.1
Pre-mRNA splicing factor activity	9416	DDX23	DEAD (Asp-Glu-Ala-Asp) box polypeptide 23	-1.9	-1.6
	25929	GEMIN5	Gem (nuclear organelle) associated protein 5	-2.1	-6.1
(8284, MF)	79833	GEMIN6	Gem (nuclear organelle) associated protein 6	-3.0	-4.6
	84967	LSM10	U7 snRNP-specific Sm-like protein LSM10	-1.5	-2.5
	57819	LSM2	LSM2 homolog, U6 small nuclear RNA associated (*S. cerevisiae*)	-1.5	-3.2
	23658	LSM5	LSM5 homolog, U6 small nuclear RNA associated (*S. cerevisiae*)	-1.4	-2.0
	51691	LSM8	LSM8 homolog, U6 small nuclear RNA associated (*S. cerevisiae*)	-1.7	-1.9
	8559	PRPF18	PRP18 pre-mRNA processing factor 18 homolog (yeast)	-3.2	-4.3
	9128	PRPF4	PRP4 pre-mRNA processing factor 4 homolog (yeast)	-2.0	-5.3
	6426	SFRS1	Splicing factor, arginine/serine-rich 1 (splicing factor 2)	-1.6	-3.2
	9169	SFRS2IP	Splicing factor, arginine/serine-rich 2, interacting protein	-1.5	-2.1
	6432	SFRS7	Splicing factor, arginine/serine-rich 7, 35kDa	-1.4	-3.7
	8487	SIP1	Survival of motor neuron protein interacting protein 1	-2.0	-4.3
RNA polymerase complex	5433	POLR2D	Polymerase (RNA) II (DNA directed) polypeptide D	-2.1	-3.2
(30880, CC)	5438	POLR2I	Polymerase (RNA) II (DNA directed) polypeptide I	-1.5	-2.8
	51728	POLR3K	Polymerase (RNA) III (DNA directed) polypeptide K	-1.6	-2.5
	10622	RPC32	Polymerase (RNA) III (DNA directed) (32kD)	-1.5	-2.6
	171568	RPC8	RNA polymerase III subunit RPC8	-1.9	-3.5
	9169	SFRS2IP	Splicing factor, arginine/serine-rich 2, interacting protein	-1.5	-2.1
	23528	ZNF281	Zinc finger protein 281	-1.5	-2.0
rRNA transcription	2971	GTF3A	General transcription factor IIIA	-1.7	-2.1
(9303, BP)	84365	MKI67IP	MKI67 (FHA domain) interacting nucleolar phosphoprotein	-1.7	-1.9
rRNA processing	10436	C2F	C2f protein	-1.5	-3.7
(6364, BP)	54606	DDX56	DEAD (Asp-Glu-Ala-Asp) box polypeptide 56	-1.4	-4.0
	29960	FTSJ2	FtsJ homolog 2 (*E. coli*)	-1.7	-2.3
	50628	GEMIN4	Gem (nuclear organelle) associated protein 4	-3.2	-10.6
	10528	NOL5A	Nucleolar protein 5A (56kDa with KKE/D repeat)	-1.4	-1.7
	9221	NOLC1	Nucleolar and coiled-body phosphoprotein 1	-1.4	-1.4

	22984	PDCD11	Programmed cell death 11	-1.5	-2.6
	23404	RRP4	Homolog of Yeast RRP4 (ribosomal RNA processing 4), 3'-5'-exoribonuclease	-1.9	-3.7
	51010	RRP40	Exosome component Rrp40	-2.1	-1.9
	54512	RRP41	Exosome complex exonuclease RRP41	-2.5	-18.4
Transcription from Pol III promoter (6383, BP)	672	BRCA1	Breast cancer 1, early onset	-1.5	-2.3
	2971	GTF3A	General transcription factor IIIA	-1.7	-2.1
	10625	IVNS1ABP	Influenza virus NS1A binding protein	-1.7	-5.7
	51728	POLR3K	Polymerase (RNA) III (DNA directed) polypeptide K	-1.6	-2.5
	10622	RPC32	Polymerase (RNA) III (DNA directed) (32kD)	-1.5	-2.6
	171568	RPC8	RNA polymerase III subunit RPC8	-1.9	-3.5
	6619	SNAPC3	Small nuclear RNA activating complex, polypeptide 3	-1.4	-2.0
	10302	SNAPC5	Small nuclear RNA activating complex, polypeptide 5	-1.6	-1.7
tRNA processing (8033, BP)	81627	C1orf25	Chromosome 1 open reading frame 25	-2.1	-3.2
	83480	FKSG32	Hypothetical protein FKSG32	-1.4	-2.1
	79042	LENG5	Leukocyte receptor cluster (LRC) member 5	-1.4	-2.1
	80746	MGC2776	Hypothetical protein MGC2776	-2.0	-3.0
	10940	POP1	Processing of precursors 1	-1.7	-3.2
	80324	PUS1	Pseudouridylate synthase 1	-1.5	-2.0
	11102	RPP14	Ribonuclease P (14kD)	-1.6	-1.4
	10248	RPP20	POP7 (processing of precursor, *S. cerevisiae*) homolog	-1.6	-3.2
	10557	RPP38	Ribonuclease P (38kD)	-1.6	-1.7
	51095	TRNT1	tRNA nucleotidyl transferase, CCA-adding, 1	-1.6	-2.5
	10785	WDR4	WD repeat domain 4	-1.6	-2.1
tRNA-specific ribonuclease activity (4549, MF)	79042	LENG5	Leukocyte receptor cluster (LRC) member 5	-1.4	-2.1
	80746	MGC2776	Hypothetical protein MGC2776	-2.0	-3.0
	10940	POP1	Processing of precursors 1	-1.7	-3.2
	11102	RPP14	Ribonuclease P (14kD)	-1.6	-1.4
	10248	RPP20	POP7 (processing of precursor, *S. cerevisiae*) homolog	-1.6	-3.2
	10557	RPP38	Ribonuclease P (38kD)	-1.6	-1.7
Mitochondrial metabolism					
Protein-mitochondrial targeting (6626, BP)	26515	FXC1	Fracture callus 1 homolog (rat)	-1.7	-2.6
	80273	GRPEL1	GrpE-like 1, mitochondrial (*E. coli*)	-1.4	-2.0
	3329	HSPD1	Heat shock 60kDa protein 1 (chaperonin)	-1.7	-4.0
	1678	TIMM8A	Translocase of inner mitochondrial membrane 8 homolog A	-2.6	-3.7
	26521	TIMM8B	Translocase of inner mitochondrial membrane 8 homolog B	-1.5	-2.0
	26520	TIMM9	Translocase of inner mitochondrial membrane 9 homolog	-1.4	-1.6
	51095	TRNT1	tRNA nucleotidyl transferase, CCA-adding, 1	-1.6	-2.5
Protein translocase activity (15450, MF)	51300	C3orf1	Chromosome 3 open reading frame 1	-1.5	-2.0
	26515	FXC1	Fracture callus 1 homolog (rat)	-1.7	-2.6
	55176	SEC61A2	Sec61 alpha 2 subunit (*S. cerevisiae*)	-1.4	-1.5
	1678	TIMM8A	Translocase of inner mitochondrial membrane 8 homolog A	-2.6	-3.7
	26521	TIMM8B	Translocase of inner mitochondrial membrane 8 homolog B	-1.5	-2.0
	26520	TIMM9	Translocase of inner mitochondrial membrane 9 homolog	-1.4	-1.6
Carnitine O-acyltransferase activity (16406, MF)	1375	CPT1B	Carnitine palmitoyltransferase 1B (muscle)	-1.7	-1.9
	1376	CPT2	Carnitine palmitoyltransferase II	-2.0	-7.0
	54677	CROT	Carnitine O-octanoyltransferase	-1.7	-1.7

The formation of autophagic vacuoles is illustrated by the GO terms "small GTPase mediated signal transduction" and "ARF guanyl-nucleotide exchange factor activity", which comprise upregulated genes involved in the control of vesicular transport and membrane trafficking, especially in the endosomal/lysosomal pathway, as for example Rab proteins [29]. In contrast, genes regulating rather exocytotic transport processes appear decreased under the term "Golgi stack". In response to amino acid starvation, cells seem to

upregulate in particular genes involved in amino acid transport. This has been previously described in yeast [30]. Amino acid-dependent metabolic and proliferative activities can be expected to be reduced to a minimum under these conditions. This is reflected in the downregulation of various genes implicated in protein and nucleic acid synthesis. Gene categories such as DNA replication and repair, ribosome biogenesis and assembly, or rRNA, tRNA, and mRNA transcription and processing among the downregulated biological functions demonstrate this phenomenon. Consequently, genes mediating cell cycle arrest are upregulated. Enduring starvation may ultimately lead to cell death. Autophagic phenotypes have been associated with programmed cell death other than apoptosis, suggesting a particular autophagic death pathway independent of caspase activation [31-33]. We observed an upregulation of genes related to apoptosis during starvation, most of which are classified in the context of apoptosis inhibition, thus supporting the model of autophagic death independent of apoptosis. Interestingly, ceramide has been described as an important mediator of autophagy and autophagic cell death [34, 35] and the GO terms "sphingoid metabolism" and "ceramide metabolism" appeared among the upregulated categories in our experiment.

Autophagy promotes the presentation of peptides from intracellular and lysosomal source proteins on MHC-II molecules

To determine whether autophagy contributes to the endogenous presentation of intracellular antigens on HLA class II in general or if this process represents a minor event followed only by some model antigens [9-11], the presentation levels of peptides from different inherent source proteins were quantified and compared between starved cells (6 h and 24 h) undergoing autophagy and non-starved control cells by mass spectrometry. For the quantitation experiments, smaller cell numbers were used, resulting in a smaller subset of MHC-II ligands that could be analyzed. In order to exclude possible influences caused by an altered MHC surface expression, we measured MHC-I and -II levels by flow cytometry and observed no substantial changes upon autophagy induction (data not shown).

Table 2.3.1.5. Differential Presentation of peptides on HLA-DR molecules and corresponding mRNA data. The given peptide and mRNA ratios refer to the comparison of cells grown under starvation with control cells. For peptides, ratios were calculated from the signal intensities in LC-MS experiments. mRNA ratios were calculated from the signal log ratios given by the microarray analysis. "NC" (= no change) is displayed if no significant change in the expression level was observed according to the change algorithm.

Source Protein	Entrez Gene ID	Peptide Sequence	6 h Starvation		24 h Starvation	
			Peptide Ratio	mRNA Ratio	Peptide Ratio	mRNA Ratio
Membrane Proteins						
HLA-A*0201	3105	FVRFDSDAASQR	0.64	NC	1.53	NC
	3105	FVRFDSDAASQRME	0.68	NC	1.30	NC
	3105	DTQFVRFDSDAASQRME	1.58	NC	0.36	NC
	3105	VDDTQFVRFDSDAASQR	1.15	NC	0.82	NC
	3105	KHKWEAAHVAEQLR	1.09	NC	1.23	NC
	3105	DDTQFVRFDSDAASQRME	1.18	NC	0.95	NC
HLA-B*4402	3106	EDLSSWTAADTAAQITQRKWE	1.18	NC	0.42	NC
	3106	LSSWTAADTAAQITQR	1.11	NC	1.07	NC
HLA-Cw*0501	3107	VDDTQFVQFDSDAASPRGEPR	1.20	NC	0.50	NC
	3107	KDYIALNEDLRSWTA	1.11	NC	-	NC
	3107	DGKDYIALNEDLRSWTA	1.01	NC	0.61	NC
	3107	FVQFDSDAASPRGEPR	0.76	NC	1.12	NC
HLA-E	3133	DLRSWTAVDTAAQISEQ	0.97	1.87	0.70	2.46
HLA-DQB1*0301	3119	DVEVYRAVTPLGPPD	1.25	NC	-	NC
lymphocyte antigen Ly-6E	4061	KPTICSDQDNYCVT	1.18	NC	-	0.54
	4061	LKPTICCSDQDNYCVT	1.40	NC	0.97	0.54
immunoglobulin heavy chain	3492	YLQMNSLKTEDT	0.75	-	1.33	
	3492	TLYLQMNSLKTEDT	1.38	-	-	
immunoglobulin lambda chain	3537	SHKSYSCQVTHEGSTVE	1.02	-	1.45	
B-lymphocyte antigen CD 20	931	INIYNCEPANPSEK	1.16	NC	1.53	NC
class I cytokine receptor	9466	VGVPYRITVTAVSASG	1.20	NC	-	NC
transferrin receptor protein 1	7037	FTYINLDKAVLGTSN	1.18	NC	0.85	NC
carboxypeptidase D	1362	VPGTYKITASARGYNPV	1.27	1.23	1.37	1.52
	1362	VPGTYKITASARGYN	1.13	1.23	-	1.52
Extracellular Proteins						
serotransferrin [bovine]		FVKDQTVIQNTD	0.66	-	1.37	-
		DVAFVKDQTVIQNTD	1.13	-	-	-
		DVAFVKDQTVIQ	1.24	-	-	-
serum albumin [bovine]		SPDLPKLKPDPNTLCDEF	1.24	-	1.01	-
apolipoprot B-100 [bovine]		SASYKADTVAKVQGT	1.08	-	1.02	-
		SASYKADTVAKVQGTE	0.98	-	0.44	-
Intracellular Proteins						
heat shock 70 kDa protein 1	3303	NVLRIINEPTAAAIAYG	1.50	3.48	1.48	NC
	3303	VLRIINEPTAAAIAY	1.03	3.48	1.24	NC
	3303	RIINEPTAAAIA	1.49	3.48	2.25	NC
	3303	VLRIINEPTAAAIAYG	1.12	3.48	1.30	NC
heat shock cognate 71 kDa protein	3312	GILNVSAVDKSTGKE	1.67	NC	1.51	NC
	3312	ERAMTKDNNLLGKFE	1.19	NC	1.50	NC
	3312	GERAMTKDNNLLGKFE	1.48	NC	1.30	NC
elongation factor 1-alpha 1	1917	IEKFEKEAAEMGKGSF	1.49	NC	2.87	NC
TNF, alpha induced protein 3	7128	EIIHKALIDRNIQ	1.32	2.14	-	2.64
RAD23 homolog B	5887	LLQQISQHQEHF	1.88	NC	1.79	NC
actin, cytoplasmic 2	71	TDYLMKILTERGYS	1.30	NC	1.09	NC
NEDD4La	23327	DGRTFYIDHNSKITQ	1.26	NC	1.51	NC
T-complex protein 1, beta subunit	10576	SLMVTNDGATILKN	1.15	NC	-	NC
ubiquitin	7311	SDYNIQKESTLHLV	1.05	-	1.42	-
alpha enolase	2023	VPLYRHIADLAGNSEV	1.50	NC	1.14	NC
syntaxin 6	10228	NPRKFNLDATELSIRK	1.60	NC	-	NC
tubulin beta-5 chain	10382	EPYNATLSVHQL	1.50	NC	1.23	NC
Lysosomal Proteins						
cathepsin C	1075	YDHNFVKAINAIQKSWT	1.31	NC	1.28	NC
	1075	YDHNFVKAINAIQKSW	1.28	NC	1.27	NC
	1075	YDHNFVKAINAIQKS	1.56	NC	1.40	NC
cathepsin D	1509	LSRDPDAQPGGE	0.83	NC	2.30	NC
cathepsin S	1520	TTAFQYIIDNKGIDSD	1.61	2.30	-	4.92
	1520	TTAFQYIIDNKGID	1.90	2.30	1.56	4.92
lysosomal alpha-mannosidase	4125	VDYFLNVATAQGRYY	1.64	NC	-	NC

Fifty-four HLA-DR-bound peptides from 31 different source proteins were sequenced, quantified, and divided into two groups: peptides from membrane and secreted proteins, which should be preferentially presented on MHC-II

molecules, and peptides from intracellular, especially nuclear, proteins, which should be preferentially presented on MHC-I molecules (Table 2.3.1.5). Additionally, we analyzed peptides from lysosomal proteins, because lysosomes take part in the autophagic turnover of the cell. After 6 h starvation, the presentation of peptides from intracellular and lysosomal proteins rose on average by 27% and after 24 h starvation by 56% (Figure 2.3.1.4) compared with peptides from membrane and secreted proteins. Upon application of unpaired two-tail student´s t-tests to the two groups of quantified ligands, the means turned out to be significantly different ($p<0.001$) with non-overlapping 99% confidence intervals.

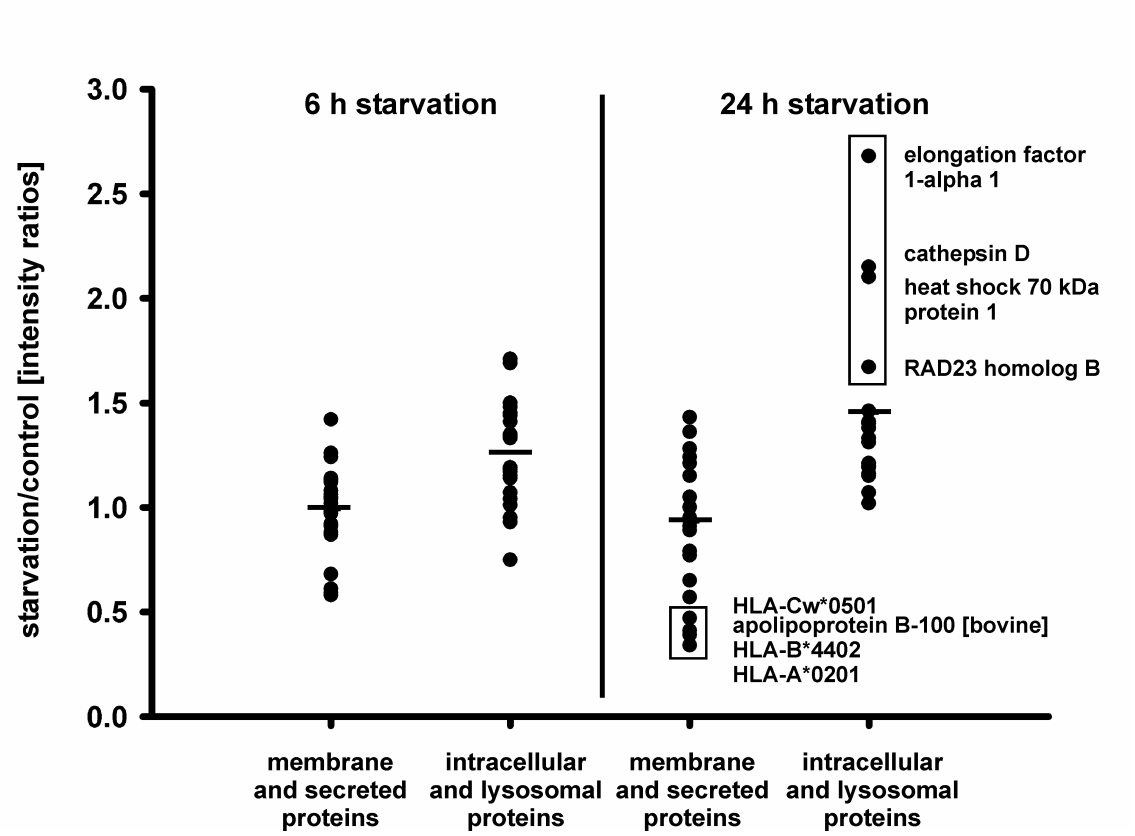


Figure 2.3.1.4. Altered peptide presentation on HLA-DR under starvation. Displayed are the relative intensity ratios of HLA-DR eluted peptides from starved cells (6 h and 24 h) and control cells as assessed by liquid chromatography (LC)-mass spectrometry (MS). Peptides were quantified by their relative peak heights in mass spectra and grouped according to the cellular localization of their source proteins: membrane plus secreted proteins and intracellular plus lysosomal proteins. Data of serial LC-MS runs were normalized to the abundant peptide LSSWTAADTAAQITQR, which showed only marginal differences in presentation levels (Table 2.3.1.5). Horizontal bars indicate the mean intensity ratios for each group. Marked in a box are the 4 peptides that showed the highest presentation levels after 24 h starvation. Their source proteins are localized in the nucleus and in lysosomes.

Enhancement of presentation appeared to be selective for the cellular localization of peptide source proteins. From the 4 source proteins that showed

the highest presentation levels of peptides after 24 h starvation, 3 are localized in the nucleus and 1 in lysosomes (Table 2.3.1.5, Figure 2.3.1.4). In the case of these 4 peptides, presentation levels were raised on average by 131% after 24 h autophagy. These changes represent relative differences in presentation levels normalized to the abundant peptide LSSWTAADTAAQITQR (Table 2.3.1.5). The absolute changes in presentation levels could not be assessed by our assay.

Apart from an increased uptake into autophagic vacuoles, several other processes might contribute to an enhanced presentation of peptides derived from intracellular proteins under starvation. To examine whether a higher mRNA expression for specific proteins upon autophagy induction led to an increased peptide presentation, gene expression for all 31 source proteins was assessed by oligonucleotide microarrays (Table 2.3.1.5). On average, mRNA levels of most genes remained stable under starvation. Among the membrane proteins, only HLA-E and carboxypeptidase D displayed an increased expression. For intracellular and lysosomal proteins, the same could be observed for TNF alpha-induced protein 3, heat shock 70 kDa protein 1, and cathepsin S. Peptides from the corresponding source proteins were also presented in higher amounts at the cell surface after induction of autophagy. We therefore cannot exclude the possibility that overexpression of these particular proteins during autophagy was the reason for elevated presentation levels of the corresponding peptides at the cell surface. However, only intracellular source proteins from 7 of 24 analyzed peptides showed elevated mRNA expression levels during autophagy. It is therefore highly unlikely that altered source gene expression was a major contributor to the observed changes in presentation levels.

In addition, an enhanced presentation of intracellular peptides on MHC-II molecules on cells undergoing autophagy might be due to an enhanced uptake of cellular debris by live cells, although this should affect intracellular and membrane proteins similarly. To exclude this possibility we incubated control cells and cells undergoing autophagy with the corresponding amounts of dead cells (3 freeze-thaw rounds) and analyzed the MHC-II ligands as described. We observed no enhanced presentation of intracellular peptides if dead cells were present (data not shown). Therefore, an enhanced uptake of dead cells in the starved samples does not contribute to the observed changes in MHC-II peptide presentation levels.

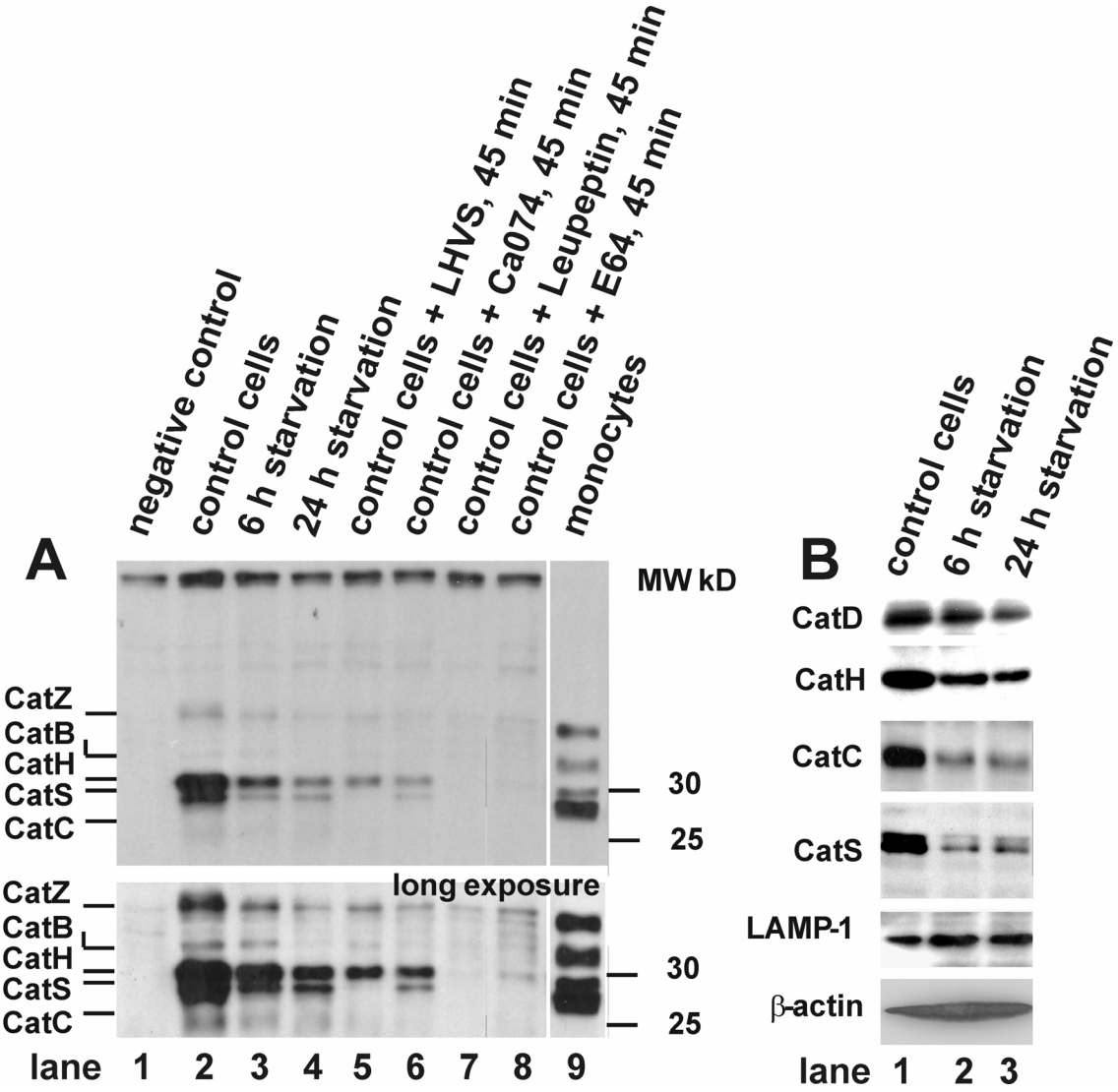


Figure 2.3.1.5. (A) Affinity labelling of active cathepsins. Endocytic extracts were generated from control cells, cells after 6 h and 24 h starvation, and from human peripheral blood monoyctes, respectively, by differential centrifugation as reported [18, 36]. 5 µg total endocytic protein (1.5 µg in monocytes) were either directly incubated with the active site-restricted biotinylated affinity label DCG-0N as described (lane 2: control cells; lane 3: 6 h starvation; lane 4: 24 h starvation; and lane 9: monocytes), or were subjected to 95°C as negative control (lane 1). In addition, control cells were incubated with the CatS-inhibitor LHVS (25 nM), the CatB-inhibitor Ca074 (1 µM), the pan-cysteine protease inhibitors leupeptin (1 mM) or E64 (25 µM) (lanes 5-8), for 45 min at 37°C prior to labelling, as further controls. Active cathepsins were visualized after resolution by SDS-PAGE by streptavidin-HRP blot: Cat Z, B, H, and S at 36, 33, 30, and 28 kDa, respectively. **(B) Cathepsin polypeptides probed by Western blot**. Identical amounts of total cellular protein from control cells (lane 1) and cells undergoing autophagy (6 h and 24 h starvation, respectively; lane 2 and lane 3) were probed for CatS, CatC, CatD, CatH, β-actin and LAMP-1 by Western blot.

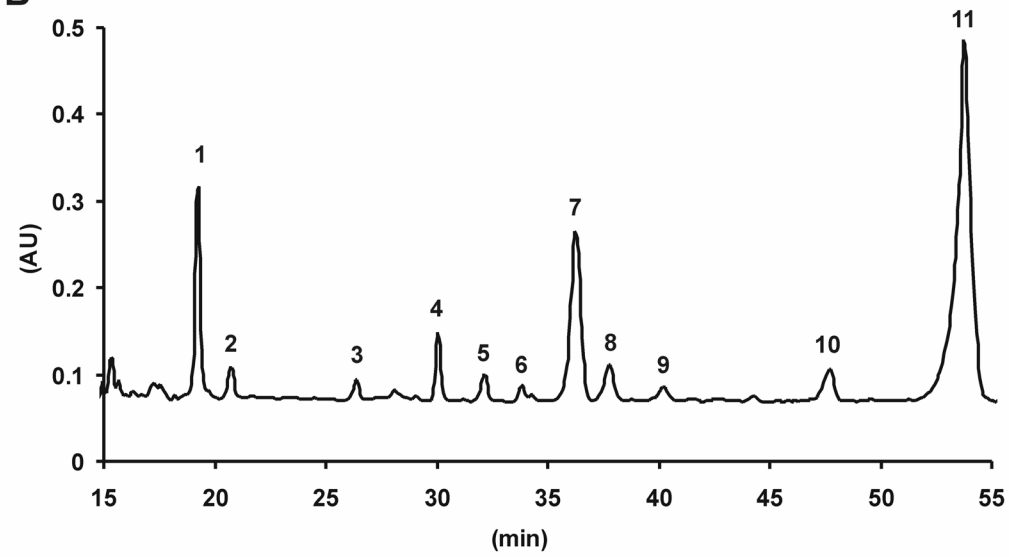
Autophagy leads to a time-dependent decrease of lysosomal proteases and altered antigen processing

Interestingly, presentation levels of peptides derived from the same source protein were differentially affected by starvation. This applied both, to proteins processed by the classical MHC-II pathway, for example HLA-A*0201, as well as to intracellular proteins, such as heat shock 70 kDa protein 1 (Table 2.3.1.5). This led us to hypothesize that activation of the autophagic pathway might concomitantly affect the MHC-II processing machinery by altering the activity levels of lysosomal proteases. Therefore, we assessed the activity of the major cathepsins during autophagy by affinity labelling (Figure 2.3.1.5A). Active cathepsins Z, B, H, S and C could be detected in control cells using this method, largely in agreement with previous studies in other cells [20]. Starvation of cells led to a time-dependent decrease of the activity signals for all cathepsins without a clear preference for any individual cathepsin. The same pattern of downregulation was observed when control cells and cells undergoing autophagy were probed for cathepsin polypeptides by Western blot (Figure 2.3.1.5B). This effect was not due to non-selective breakdown of total cellular protein or lysosomal protein in general, because the amounts of β-actin as well as of the lysosome-resident protein LAMP-1 remained unaffected by autophagy.

To assess whether cathepsin downregulation had an effect on the generation of antigenic peptides, we incubated myelin basic protein (MBP) peptide ENPVVHFFKNIVTPRTP (MBP_{83-99}), a well-characterized model peptide for MHC-II processing [37], with active lysosomal extracts from control cells and cells undergoing autophagy and analyzed the degradation products by RP-HPLC, MALDI-MS and Edman microsequencing. Figure 2.3.1.6A shows the detected cleavage points which are largely in agreement with earlier data [21]. A quantitative analysis of the degradation products (Figure 2.3.1.6B) revealed that the downregulation of lysosomal proteases by autophagy affected the breakdown products to a different extent. As expected, the amount of undigested peptide was higher in autophagic cells, which exhibited lower cathepsin levels than control cells, and, in agreement with this, most of the breakdown products were more abundant in control cells than in autophagic cells (Figure 2.3.1.6C).

A

B



C

Peak	Peptide Sequence	Protease	Amount (%)	
			Control cells	Autophagic cells
1	IVTPRTP	AEP	21.5	12.6
2	NIVTPRTP	Cat S/L	3.3	2.2
3	FKNIVTPRTP	Cat D	2.2	3.2
4	ENPVVHF	Cat D	7.0	5.0
5	NPVVHFFKN	-	2.5	0.5
6	FFKNIVTPRTP	Cat S/L	1.4	1.2
7	ENPVVHFFKN	AEP	17.2	12.9
8	ENPVVHFFK	Cat S/L	3.6	1.5
9	VVHFFKNIVTPRTP PVVHFFKNIVTPRT	-	1.3	1.2
10	NPVVHFFKNIVTPRTP	-	3.2	1.1
11	ENPVVHFFKNIVTPRTP	undigested	36.6	58.7

Figure 2.3.1.6. MBP_{83-99} digestion with lysosomal extracts from control cells and cells undergoing autophagy. (A) Preferential detected cleavage sites. MBP_{83-99} was incubated at pH 5.4 with lysosomal extracts from control cells and cells undergoing 24 h autophagy for 3 h. Breakdown products were subsequently separated by RP-HPLC and analyzed by MALDI-MS and Edman microsequencing. **(B) RP-HPLC chromatogram of control cell MBP breakdown products at 214 nm.** The annotated peaks correspond to the major breakdown products of MBP_{83-99} as identified by MALDI MS and Edman microsequencing. The corresponding chromatogram of autophagic cells is not shown. **(C) Quantitative distribution of MBP_{83-99} breakdown products.** Breakdown products were quantified by their peak heights in the RP-HPLC chromatogram. The total amount of identified peptides was set to 100%. Shown are the differences between control cells and cells undergoing 24 h autophagy and the lysosomal proteases which are known to be responsible for the generation of the corresponding fragments [21].

However, the ratios of downregulation of breakdown products differed markedly corresponding to the involved proteases. Whereas asparagine endoproteinase (AEP) appeared to be quite sensitive to autophagy – the corresponding peptides dropped at an average of 33.2% – cathepsin D seemed to be more resistant, which is in concordance with the Western blot results (Figure 2.3.1.5B). Thus, an overall downregulation of active lysosomal proteases with only subtle differences between the key enzymes can nevertheless markedly influence the generation of different MHC-II ligands corresponding to the involved proteases. This could explain the differences in MHC-II presentation levels of different peptides from the same source proteins that were observed in cells undergoing autophagy and control cells (Table 2.3.1.5).

2.3.1.5 Discussion

In order to assess the impact of autophagy on the HLA class II ligandome, we started by performing a detailed characterization of the MHC-II self peptide repertoire of a cell line grown under normal conditions. This analysis revealed that peptides from source proteins that are localized in almost all cell compartments and take part in general cellular processes are presented on MHC-II molecules. Some examples of peptides from intracellular proteins on MHC-II have been described before [38, 39]. However, in our case the number of such source proteins was surprisingly high. This possibly reflects the detected basal level of autophagy which might be responsible for a constant shuttling of intracellular source proteins into the endosomal/lysosomal compartment. Thus, peptides from intracellular antigens are likely to have a larger impact on CD4+ helper T cell regulation than was originally believed. It has already been reported that CD4+ T cells are able to recognize peptides from intracellular melanoma antigens [40, 41] and from the viral antigen EBNA1 [42], and that under inflammatory conditions peptides from intracellular antigens are presented on HLA class II molecules on epithelial cells which are target cells in autoimmunity [43].

This study demonstrates that autophagy constitutes a general pathway promoting the processing of intracellular proteins by lysosomes and presentation of the resulting peptides on MHC-II molecules. Autophagy is a constitutive process responsible for the turnover of intracellular proteins [44]. Basal levels have been observed in most tissues [45] and can be particularly

enhanced by starvation. In addition, autophagy is involved in tumor development [45, 46]. Starvation-induced autophagy has been observed for example in lymphocytes isolated from patients with chronic lymphocytic leukemia [47]. This might indicate an important role of this process in tumor survival under nutrient-limiting conditions. In contrast, autophagy as a form of programmed cell death may accelerate tumor development if it is decreased [48]. Some anticancer drugs potentially act via triggering autophagy [35] and by doing so could cause an enhanced presentation of intracellular CD4+ T cell epitopes in MHC-II-expressing tumor cells. Autophagy has also been described as a constitutive process under nutrient-rich conditions for several tissues *in vivo*, including thymic epithelial cells [49]. For this reason, it might play an important role in the presentation of intracellular self-antigens to CD4+ T cells during negative selection.

Our results indicate a profound impact of enhanced autophagy on MHC-II antigen processing caused by a decrease of active cathepsins in the endocytic compartment. Decreased cathepsin levels might favor the generation of MHC-II peptides due to a less efficient lysosomal protein digestion. This has been suggested as a mechanism to explain the superiority of dendritic cells over macrophages as antigen presenting cells [50]. Similarly, autophagy might subject the cell to an enhanced immune surveillance by CD4+ T cells under potentially dangerous stress conditions.

Recently, it has been reported that peptides from cytosolic antigens [9, 10] as well as from a cytosolic viral antigen [11] can be presented via autophagy on MHC-II molecules. It was not clear, however, if this represents a minor event or if autophagy contributes to the endogenous presentation of intracellular antigens on HLA class II in general. This study sheds more light on this issue by demonstrating that autophagy affects MHC-II presentation of peptides from intracellular proteins in general and by providing clear evidence for altered lysosomal processing. Thus, apart from its various known implications in stress responses and cell death, autophagy might play an important role in the regulation of CD4+ T cell-mediated processes.

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2.3.1.7 References

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3 Summary

Molecularly defined immunotherapies against cancer require the identification of appropriate tumor associated antigens (TAAs). Against this background, patient-individual TAAs for renal cell carcinoma were identified by whole-genome mRNA expression analysis of tumors, autologous normal kidney, and a selection of other normal tissues using oligonucleotide microarrays. These results were applied to HLA ligands simultaneously identified on the patients' tumors in order to define peptide vaccines aiming at the generation of anti-tumor cytotoxic T lymphocyte (CTL) responses. In addition, gene expression analysis was used to demonstrate that the overall expression profiles of metastases are closely related to the primary tumor. This similarity justifies the approach of TAA identification using resected primary RCC tumors in order to target metastases with the so defined vaccination cocktail.

This approach for TAA identification was also applied to colorectal carcinoma, which is a heterogeneous tumor species requiring the careful isolation of tumor cells and corresponding normal epithelial cells in order to obtain tumor cell-specific expression data. Laser microdissection followed by mRNA amplification was employed to generate microarray data and identify candidate TAAs. Impaired RNA quality is a frequent problem in this setting but it could be shown that moderate levels of degradation may still lead to meaningful microarray results.

So far, it was unclear whether MHC class II positive tumors are able to directly present peptides of TAAs to $CD4^+$ T helper cells. We could show that autophagy is a process enabling the presentation of MHC-II peptides from intracellular source proteins in general, thereby providing a mechanism for the potential recognition of tumors by $CD4^+$ T cells. These results show how TAAs on MHC-II might arise and illustrates how comprehensive gene expression analysis may contribute to the elucidation of such a fundamental process, which likely has important implications for tumor immunology and cancer immunotherapy.

Zusammenfassung

Jede molekular definierte Immuntherapie gegen Krebs erfordert die Identifizierung geeigneter tumor-assoziierter Antigene (TAAs). Vor diesem Hintergrund wurden patientenindividuelle TAAs beim Nierenzellkarzinom identifiziert. Dazu kam eine genomweite mRNA-Expressionsanalyse von Tumoren, autologem Nierennormalgewebe und einer Auswahl anderer Normalgewebe mittels Oligonukleotid-Microarrays zum Einsatz. Diese Ergebnisse wurden mit HLA-Liganden in Beziehung gesetzt, die zugleich auf Tumoren der Patienten identifiziert wurden, um Peptidvakzine zur Erzeugung einer gegen den Tumor gerichteten Immunantwort durch cytotoxische T-Lymphocyten (CTLs) zu definieren. Zusätzlich wurde mit Hilfe der Genexpressionsanalyse gezeigt, dass die Gesamtexpressionsprofile von Metastasen denen eines Primärtumors sehr ähnlich sind. Diese Ähnlichkeit rechtfertigt die Identifizierung von TAAs auf operativ entfernten Primärtumoren, um mit dem so definierten Vakzin Metastasen anzugreifen.

Diese Strategie zur Identifizierung von TAAs wurde auch auf das colorectale Karzinom angewandt. Es stellt eine heterogene Tumorspezies dar, die eine sorgfältige Isolierung von Tumor- und korrespondierenden normalen Epithelzellen erfordert, um tumorzellspezifische Daten zu ermöglichen. Deshalb wurde ein Verfahren zur lasergestützten Mikrodissektion eingesetzt, um Microarray-Daten zu erhalten und potenzielle TAAs zu identifizieren. In diesem Zusammenhang tritt häufig das Problem auf, dass die gewonnene RNA in ihrer Qualität beeinträchtigt ist. Es konnte aber gezeigt werden, dass ein gewisses Maß an RNA-Abbau immer noch zu aussagekräftigen Microarray-Daten führen kann.

Bisher war unklar, ob MHC Klasse II-positive Tumore Peptide von TAAs direkt $CD4^+$ T-Helferzellen präsentieren können. Wir konnten zeigen, dass Autophagie ein Vorgang ist, der die Präsentation von MHC II-Peptiden aus intrazellulären Quellproteinen allgemein ermöglicht. Dies könnte ein Mechanismus für die Erkennung von Tumoren durch $CD4^+$ T-Zellen sein. Diese Ergebnisse zeigen, wie TAAs auf MHC II entstehen könnten und liefern ein Beispiel dafür, wie umfangreiche Genexpressionsanalysen zur Aufklärung solch eines grundlegenden Prozesses beitragen können, der möglicherweise wichtige Auswirkungen für Tumorimmunologie und Krebsimmuntherapie haben könnte.

4 Abbreviations

Ab	antibody
ACSL4	acyl-CoA synthetase long-chain family member 4
ADCC	antibody-dependent cell-mediated cytotoxicity
ADFP	adipose differentiation-related protein, adipophilin
AEP	asparagine endoproteinase
APC	antigen presenting cell
APOL1	apolipoprotein L, 1
aRNA	antisense ribonucleic acid
BCG	bacillus Calmette-Guerin
BIRC5	baculoviral IAP repeat-containing 5 (survivin)
bp	base pairs
CA9	carbonic anhydrase IX
CatB	cathepsin B
CCA	colorectal carcinoma
CCD	charge-coupled device
CCNB1	cyclin B1
CD	cluster of differentiation
CDC2	cell division cycle 2, G1 to S and G2 to M
CDK1	= CDC2
CDK4	cyclin-dependent kinase 4
CDKN3	cyclin-dependent kinase inhibitor 3
cDNA	complementary deoxyribonucleic acid
CEA	carcinoembryonic antigen
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6
CML	chronic myelogenous leukemia
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	deoxynucleoside triphosphate
DRiPs	defective ribosomal products
dscDNA	double-stranded cDNA
DTT	dithiothreitol
EBNA1	Epstein-Barr nuclear antigen 1
EDTA	ethylene diamine tetraacetic acid
EEF2	eukaryotic translation elongation factor 2
ESI	electrospray ionization
FAT10	= UBD
Fc	constant fragment
FCS	fetal calf serum
FGF5	fibroblast growth factor 5
GCOS	GeneChip Operating Software
GM-CSF	granulocyte-macrophage colony stimulating factor
GvHD	graft-versus-host disease
GvT	graft-versus-tumor
HBSS	Hank’s Balanced Salt Solution
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HPV	human papilloma virus
HRP	horse radish peroxidase
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2
IDO	= INDO, indoleamine-pyrrole 2,3 dioxygenase
IFN	interferon
Ig	immunoglobulin
IGF1	insulin-like growth factor 1
IGFBP3	insulin-like growth factor binding protein 3
kDa	kilodalton

KLH	keyhole limpet hemocyanin
LGALS2	lectin, galactoside-binding, soluble, 2 (galectin 2)
LMM	laser microbeam microdissection
LPC	laser pressure catapulting
mAb	monoclonal antibody
MAD2	= MAD2L1, mitotic arrest deficient-like 1
MALDI	matrix-assisted laser desorption ionization
MBP	myelin basic protein
MCM	minichromosome maintenance deficient
M-CSF	macrophage colony stimulating factor
MDC	monodansylcadaverine
MHC	major histocompatibility complex
MIC	MHC class I chain–related molecule
MMP7	matrix metalloproteinase 7 (matrilysin, uterine)
MUC	mucin
NK cell	natural killer cell
NKG2D	= KLRK1, killer cell lectin-like receptor subfamily K, member 1
NKT cell	natural killer T cell
NTP	nucleoside triphosphate
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase
ORF	open reading frame
PCR	polymerase chain reaction
PSA	= KLK3, kallikrein 3, (prostate specific antigen)
qPCR	quantitative real-time RT-PCR
RCC	renal cell carcinoma
RGS	regulator of G-protein signalling
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase PCR
SDS	sodium docecyl sulfate
SLR	signal log ratio
TAA	tumor associated antigen
TCR	T cell receptor
TERT	telomerase reverse transcriptase
TGF	transforming growth factor
TGFBR2	transforming growth factor, beta receptor II
Th1 cell	T helper 1 cell
TIL	tumor infiltrating lymphocyte
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFSF7	tumor necrosis factor (ligand) superfamily, member 7, = CD70
TOF	time of flight
T_{Reg} cell	regulatory T cell
tRNA	transfer ribonculeic acid
TRP	tyrosinase related protein
UBD	ubiquitin D
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau
WT1	Wilms tumor 1

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6 Publications

1. Dengjel, J.*, **Schoor, O.***, Fischer, R., Reich, M., Kraus, M., Müller, M., Kreymborg, K., Altenberend, F., Brandenburg, J., Kalbacher, H., Brock, R., Driessen, C., Rammensee, H. G. & Stevanovic, S. (2005) From the Cover: Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. *Proc. Natl. Acad. Sci U. S. A* 102: 7922-7927.

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