Biomarker Discovery in Cutaneous Malignant Melanoma

A Study Based on Tissue Microarrays and Immunohistochemistry

MARGRÉT AGNARSDÓTTIR
Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjöldsv 20, Uppsala, Saturday, April 2, 2011 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

The incidence of cutaneous malignant melanoma has increased dramatically in Caucasians the last few decades, an increase that is partly explained by altered sun exposure habits. For the individual patient, with a localized disease, the tumor thickness of the excised lesion is the most important prognostic factor. However, there is a need to identify characteristics that can place patients in certain risk groups.

In this study, the protein expression of multiple proteins in malignant melanoma tumors was studied, with the aim of identifying potential new candidate biomarkers. Representative samples from melanoma tissues were assembled in a tissue microarray format and protein expression was detected using immunohistochemistry. Multiple cohorts were used and for a subset of proteins the expression was also analyzed in melanocytes in normal skin and in benign nevi. The immunohistochemical staining was evaluated manually and for part of the proteins also with an automated algorithm.

The protein expression of STX7 was described for the first time in tumors of the melanocytic lineage. Stronger expression of STX7 and SOX10 was seen in superficial spreading melanomas compared with nodular malignant melanomas. An inverse relationship between STX7 expression and T-stage was seen and between SOX10 expression and T-stage and Ki-67, respectively. In a population-based cohort the expression of MITF was analyzed and found to be associated with prognosis. Twenty-one potential biomarkers were analyzed using bioinformatics tools and a protein signature was identified which had a prognostic value independent of T-stage. The protein driving this signature was RBM3, a protein not previously described in malignant melanoma. Other markers included in the signature were MITF, SOX10 and Ki-67.

In conclusion, the protein expression of numerous potential biomarkers was extensively studied and a new prognostic protein panel was identified which can be of value for risk stratification.

Keywords: antibody-based proteomics, automated analysis, biomarker, immunohistochemistry, malignant melanoma, survival, tissue microarray

Margrét Agnarsdóttir, Department of Immunology, Genetics and Pathology, The Human Protein Atlas Project, Rudbecklaboratoriet, Uppsala University, SE-751 85 Uppsala, Sweden.

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There are no endings:
only new beginnings
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## Abbreviations

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<th>Description</th>
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<tr>
<td>ALM</td>
<td>Acral lentiginous melanoma</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’- diaminobenzidine</td>
</tr>
<tr>
<td>HPA</td>
<td>Human protein atlas</td>
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<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LMM</td>
<td>Lentigo maligna melanoma</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>NMM</td>
<td>Nodular malignant melanoma</td>
</tr>
<tr>
<td>PrESTs</td>
<td>Protein epitope signature tags</td>
</tr>
<tr>
<td>RBM3</td>
<td>RNA binding motif 3</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SOX10</td>
<td>SRY (sex determining region Y)-box 10</td>
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<td>SSM</td>
<td>Superficial spreading melanoma</td>
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<tr>
<td>STX7</td>
<td>Syntaxin-7</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor, lymph node, metastasis</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
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</table>
Introduction

Proteins/Proteome/Proteomics/Biomarker

Deoxyribonucleic acid (DNA) found in the nucleus of all eukaryotic cells constitutes the genome and functions as a recipe for proteins (Watson & Crick, 1953). The genome encompasses the genes and the non-coding DNA sequences. Through replication the genetic code is preserved. For protein synthesis the genetic code is transcribed to messenger ribonucleic acid (mRNA), which is used to build the respective protein through the process of translation. The building blocks of proteins are twenty different amino acids. The proteins often undergo post-translational modifications (e.g. glycosylation, phosphorylation, proteolytic cleavage) that alter their chemical properties resulting in compounds different in structure and function (Mann & Jensen, 2003). Proteins participate in e.g. immune responses, cell signaling, cell cycle and function as enzymes, hormones, structural components and in the transport and storage of different substances.

Proteome is a term used for all proteins expressed at a certain time and under defined circumstances by a genome. The proteome can refer to all proteins expressed in a particular cell, tissue or organism. The word is a blend of “protein” and “genome”.

Proteomics is a term used to encompass the large-scale study of proteins, particularly their structure and function. Today 21,077 protein-coding genes are known (Ensembl; Flicek et al, 2010). Similarly, genomics is the term used for the large-scale study of the genome and transcriptomics for the comprehensive study of the transcriptome (the expression levels of mRNAs). These are research fields that have exploded following the complete sequencing of the human genome (Consortium, 2004) and constitute the –omics revolution.

Protein research can be divided into two main strategies, i.e. separation-based and probe-based techniques. In the former group are methods that separate proteins on a matrix with electrophoresis but this separation is based on protein mass and isoelectric point. The latter group depends on antibodies binding to specific antigens and include immunohistochemistry (IHC) (see below), Western blot (see below) and enzyme-linked immunosorbent assay (ELISA). Antibody-based proteomics is a term used when protein-specific antibodies are used to explore the proteome (Uhlen & Ponten, 2005).
Biomarker is a characteristic that can be measured objectively and used in screening, diagnosis or follow-up of a particular disease. It can be used for treatment selection, measure response to therapy, determine prognosis or to indicate a normal biological state (Biomarkers Definitions Working Group, 2001). A protein measured in blood (e.g. prostate specific antigen (PSA)) or tissue (e.g. estrogen and progesterone receptors in breast cancer), measurements of temperature or blood pressure are examples of biomarkers with different properties. The term is rather new but in reality biomarkers have been used in medicine for a long time. For tumor tissues the tumor cells themselves usually produce the particular biomarker.

Tissue microarray

In routine histopathology tissue samples are collected after formalin-fixation. Thereafter the samples are embedded in paraffin and subsequently cut in 4-5\(\mu\)m thick sections that are laid on glass slides to be stained, most often with haematoxylin-eosin. After this process the tissue samples can be visualized and evaluated in a light microscope. A tissue microarray (TMA) allows for the rapid evaluation of multitude of tissue samples on the same glass slide. This involves collection of representative tissue cylinders from the original paraffin tissue block. The tissue cylinders are subsequently transferred to a recipient paraffin block with a consistent cylinder depth using a manual or automated tissue microarrayer (Kononen et al, 1998). The tissue cylinders are available in different sizes, 0.6-2mm. For small lesions, with sparse tissue material, the smallest cylinder is suitable to use to prevent emptying the tissue resource and often only one small cylinder can be transferred to the recipient block. The recipient block can contain more than 100 tissue cylinders that can be analyzed in various ways, e.g. with IHC (see below and Fig. 1). The consistent cylinder depth is crucial as it determines how many representative sections can be obtained from the complete array. Usually the depth is 3mm and if 4\(\mu\)m sections are taken that results in over 200 sections from each array (Kampf et al, 2004; Rimm et al, 2001). In reality the depth of the original paraffin block is often less than 3mm, which results in missing cylinders the deeper the recipient block is cut, a problem that is more apparent in studies involving small lesions.

In this thesis the TMAs are solely analyzed with IHC (see below) to detect changes at protein level but depending on the focus of the particular research project TMAs can also be analyzed in other ways to detect changes at DNA level with fluorescence in situ hybridization (FISH) or at RNA level with RNA in situ hybridization (Bubendorf et al, 1999; Moch et al, 2001; Moch et al, 1999). The material employed to construct a TMA also varies, in this thesis the material is composed of cylinders from paraffin embedded tissues but cultured cells, frozen tissues and protein arrays can also be con-
structured (Miyaji et al., 2002; Moskaluk & Stoler, 2002; Schoenberg Fejzo & Slamon, 2001).

Whatever material is used to construct an array the composition of that material can vary. The array can be used to detect new prognostic markers as in this thesis and therefore clinical follow-up information is necessary. It can also be used to detect alterations in a particular factor as a tumor progresses or screen multitude of different samples to detect alterations in a particular factor.

**Figure 1.** Generation of a tissue microarray: This involves collection of representative tissue cylinders from the original paraffin tissue block. The tissue cylinders are subsequently transferred to a recipient paraffin block. From that block four to five μm thick tissue sections are cut and laid on glass slides that can be analyzed in various ways, e.g. with immunohistochemistry.

**Figure 2.** A schematic illustration of the immunohistochemical staining procedure with 3,3’- diaminobenzidine (DAB) as a chromogen. A primary antibody that recognizes a specific tissue antigen is added and subsequently a secondary antibody coupled to horseradish peroxidase (HRP). A colorless chromogen (DAB) is converted to a brown end product after adding hydrogen peroxidase (H₂O₂).
Immunohistochemistry

IHC is widely used in routine histopathology as a complementary tool in the diagnosis of different lesions (Warford et al., 2004). This technique relies on an antibody recognizing a specific antigen and through the process of IHC the presence of this particular protein can be visualized on a glass slide containing the tissue sample. Compared with separation-based proteomic techniques this method preserves the tissue morphology, which is of great value.

Traditionally histopathological tissue samples are fixed in neutral-buffered formalin (10%) containing 4% formaldehyde that preserves the morphology well. The fixation is based on a cross-linking mechanism but the exact sites involved are unknown (Helander, 1994; Mason & O'Leary, 1991). Unfortunately the fixation process affects the site where an antibody binds to an antigen (the epitope). With IHC where the tissue samples are deparaffinized and treated in a specific way involving heat the epitopes can be unmasked (the so-called epitope retrieval) (Shi et al., 1995). Thereafter a primary antibody can be added to the slide. For the protein detection different systems are available. In this thesis the detection system involves binding a secondary antibody, labeled with an enzyme called horseradish peroxidase, to the primary one. The following visualization process relies on an enzyme-substrate reaction, which converts a colorless chromogen called 3,3′-diaminobenzidine (DAB) into a brown end product after adding hydrogen peroxide (H₂O₂) (Fig. 2). This color signal is evaluated by light microscopy of the glass slide indicating a successful antibody-antigen binding. Other detection systems are available, e.g. employing aminoethylcarbazole as a chromogen resulting in a red color end product.

There are several confounders related to this method that should be mentioned. The IHC staining results are dependent on various factors, like the specificity and sensitivity of the antibody, fixation of the tissue and on what antigen retrieval method and detection system is employed (Leong & Gilham, 1989; Paavilainen et al., 2010). Therefore it is important to standardize the staining procedure as much as possible. In addition, the evaluation is subjective and therefore lacks reproducibility, (both intra- and inter-observer) which can cause problems.

Western blot and immunofluorescence

Western blot and immunofluorescence (IF) are examples of other probe-based protein research techniques. Western blot is a technique used to detect a particular protein in a protein mixture extracted from a cell or a tissue sample (Burnette, 1981). The proteins are first separated depending on their weight with gel electrophoresis (usually sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)). Thereafter they are transferred onto
a membrane (usually nitroceullose or polyvinylidene difluoride (PVDF)) and an antibody binding to the protein of interest is added. In that way the particular protein and its predicted size can be visualized.

IF is a detection technique where fluorophores are coupled to the secondary antibody. This technique is most often used for cells or cell lines. Together with markers that identify specific organelles of the cell (e.g. cell nucleus, cytoskeleton) the protein of interest can be visualized and its subcellular localization as well (Barbe et al, 2008). Confocal microscopy is used for this type of protein detection.

The Human protein atlas project

The aim of the Human protein atlas (HPA) project is threefold: To produce validated monospecific antibodies to all proteins of the human body, to employ these antibodies to set up a database which describes where and how the proteins are expressed in tissues (with IHC) and in cells (with IF) and to employ the generated antibodies and expression data to identify potential biomarkers, especially in the field of cancer biomarkers (Berglund et al, 2008; Uhlen et al, 2005; Uhlen et al, 2010).

Monospecific antibodies are produced after immunization of rabbits with small recombinant protein fragments (100-150 amino acids) called protein epitope signature tags (PrESTs) that contain a unique area of the protein in question (Agaton et al, 2003; Nilsson et al, 2005). The selected PrESTs show a low homology for other protein coding sequences of the genome and therefore cross-reactivity of the generated antibody is minimal. The generated antibody is purified in a three-step manner and the binding specificity determined by testing the antibody on a protein array, which contains 384 different PrESTs, including the corresponding PrEST. Approved antibodies are further analyzed by Western blot using human plasma and protein lysates from two human cell lines (RT4, U251mg), human liver and tonsil. Subsequently the antibodies are stained on microarrays using IHC. The protein expression in different tissues and cells is thereafter evaluated and the results published in an open web-based protein atlas (www.proteinatlas.org). In the current version of the atlas (version 7.1) the protein expression of more than 13150 antibodies is presented. They target proteins from more than 10100 human genes corresponding to about 50% of the human protein-coding genes.

The TMAs in the HPA project include 46 selected normal tissue types and 216 tumors representing 20 different tumor types. In addition, 47 cell lines and 9 samples of primary blood cells are included in a cell microarray format (Andersson et al, 2006). Among the tumor tissues are 12 cases of cutaneous malignant melanoma (see below) with two tissue cylinders from every case. This high-throughput method allows for the identification of proteins that are of interest to study further in larger patient cohorts.
Melanocytic lesions

Melanocytes are cells that are specialized to synthesize and transfer a photo protective pigment called melanin to keratinocytes in the epidermis of the skin by using dendritic processes (Tolleson, 2005). Melanocytes are derived from melanoblasts, which are derived from the neural crest, a temporary structure formed very early in the developing embryo when the neural tube is closing. SRY (sex determining region Y)-box 10 (SOX10) (Kelsh, 2006; Wright et al, 1993) and microphthalmia-associated transcription factor (MITF) (Hodgkinsson et al, 1993; Widlund & Fisher, 2003), which is under the control of SOX10, are important for the differentiation of melanoblasts and their survival. The neural crest cells give rise to cells with migratory potential along definitive pathways in the developing embryo forming nerves and glia of the peripheral nerve system, melanocytes and craniofacial cartilage and bone in the head (Dupin & Le Douarin, 2003).

Melanocytes are most abundant in the skin but melanocytes are also found in the eye and leptomeninges of the brain. In the skin they are evenly distributed in the basal layer of the epidermis as solitary cells. They are also found in hair follicles and are in that way responsible for hair color. Melanocytes located in the skin can form different melanocytic lesions where the most common are benign melanocytic nevi and dysplastic nevi. The malignant form is cutaneous malignant melanoma, which is the subject of this thesis. Malignant melanoma can also arise in the eye, meninges and in various mucosal surfaces (e.g. anorectal, head and neck region) (Laver et al, 2010; Liubinas et al, 2010; Seetharamu et al, 2010).

Benign nevi are most often acquired during childhood and early adulthood, with increasing age the nevi disappear (Cooke et al, 1985; Gallagher et al, 1990). A minority (1-3%) are congenital nevi (Boccardi et al, 2007; Karvonen et al, 1992; Walton et al, 1976). Benign acquired nevi are divided into three different subtypes depending on the microscopical appearance. They can be junctional with melanocytic nests located at the junction between the epidermis and the dermis, compound with melanocytes both in the junctional area and in the dermis and dermal with melanocytes only found in the dermis. In benign nevi no mitoses are found in the dermis and the cells located deep in the dermis are smaller than those located higher up, a sign of differentiation.

Melanocytes can also form dysplastic pigment nevi that have a more varied macro- and microscopical appearance compared with the benign variant.
Dysplastic nevi were first described in individuals belonging to families that were prone to develop malignant melanoma (Clark et al., 1978; Lynch et al., 1978). Dysplastic nevi are associated with increased risk for developing malignant melanoma where the risk increases with larger numbers of dysplastic nevi (Tucker et al., 1997). However, the majority of malignant melanomas develop in normal skin (Bevona et al., 2003). In the light microscope the diagnosis of a dysplastic nevus is based on both cytological and architectural features (Mooi, 1997). The melanocytes are found as individual cells spread in a lentiginous pattern and as melanocytic nests that vary in size. These nests often lie horizontally with bridging of adjacent rete ridges. In the dermis there is increased fibrosis and chronic inflammation. The cellular atypia varies but for cases with severe atypia, architectural changes and pagetoid spread of atypical melanocytes in the epidermis, malignant melanoma \textit{in situ} has to be considered. Other melanocytic tumors are e.g. the blue nevus and the Spitz nevus, which are often seen in routine clinical pathology.
Malignant melanoma

Incidence
Malignant melanoma is the leading cause of skin-related deaths in Caucasians but malignant melanoma seldom affects colored individuals. The incidence has increased dramatically the last few decades with an almost four-fold increase in all the Nordic countries in the time period 1964-2003 (Tryggvadottir et al, 2010). In 1970 the age-standardized incidence rate in Sweden was less than 10/100,000 compared with 25.3/100,000 for men and 25.7/100,000 for women in the year 2007 (SOS).

Risk factors
The increased incidence is mainly explained by altered sun exposure habits. The ultraviolet radiation is harmful to the skin as it damages the cells and influences the immune system. Short intermittent exposure with sunburns is a more risky behavior than constant exposure for longer time periods and increased risk is also associated with sunburns in childhood and tanning bed use (Narayanan et al, 2010; Rigel, 2010). Other important risk factors, which are hereditary, are the number of pigment nevi, the number of dysplastic nevi and skin type (Gandini et al, 2005a; Gandini et al, 2005b; Gandini et al, 2005c). An increased risk is coupled to many nevi, both benign and dysplastic and fair skin. Familial history of malignant melanomas is a risk factor but approximately 8-12% of malignant melanoma cases are familial (Manson et al, 2000).

Diagnosis
The so-called ABCD criteria have been widely used in the recognition of malignant melanoma (Friedman & Rigel, 1985). These criteria stand for asymmetry, border irregularity, color variegation and diameter (Fig. 3). Later on an expansion of the criteria to ABCDE was proposed to include evolving and in that way include lesions that change over time. Surface microscopy (dermatoscopy) aids also in the diagnosis. The golden standard is histopathological examination of the excised lesion.
Figure 3. A malignant melanoma tumor on the skin surface. The tumor is asymmetric with irregular borders and color variegation (from http://skincancer-fact.com).

Figure 4. Microscopical pictures demonstrating that malignant melanoma tumors often look very different in the light microscope, A. Large cells with abundant cytoplasm and distinct nucleoli, B. Smaller cells with enlarged nuclei, C. A tumor with abundant melanin (the brown pigment).

Malignant melanoma subtypes

Infiltrative malignant melanoma is traditionally divided into four principal subtypes based on the microscopical appearance. The subtypes are called: superficial spreading melanoma (SSM), nodular malignant melanoma (NMM), lentigo maligna melanoma (LMM) and acral lentiginous melanoma (ALM) (Clark et al, 1969; Clark & Mihm, 1969). If the atypical melanocytes are only located above the basal membrane the lesion is diagnosed as malignant melanoma in situ.

Some of the subtypes evolve through different phases of tumor progression, which are called radial growth phase and vertical growth phase. The radial growth phase is characterized by pagetoid spread of atypical melanocytes in the epidermis which means that atypical melanocytes are located high up in the epidermis, not only basally where the melanocytes are normally located. As part of the radial growth phase small nests in the superficial papillary dermis can be seen without any mitotic activity. The vertical growth phase is characterized by nests of melanocytes in the dermis but the nests are larger than those found in the epidermis and in addition mitotic
activity can be seen in the dermal nests. The distinction between these different growth phases is important, as the tumor doesn’t have the potential to metastasize until the vertical growth phase has developed (Clark et al, 1975; Guerry et al, 1993; Herlyn et al, 1985).

Superficial spreading melanoma
SSM is the most common subtype in Caucasian (approx. 60% of infiltrative melanomas). The incidence of SSM has increased proportionally more than the other subtypes and the tumors are diagnosed at an earlier stage (Lipsker et al, 1999; Thorn et al, 1994). SSM is characterized in the beginning by extensive radial growth phase that can later evolve into the vertical growth phase with infiltration into the dermis.

Nodular malignant melanoma
NMM is the second most common subtype (approx. 20% of infiltrative melanomas) that only has a vertical growth phase. These tumors are therefore more often thicker and more advanced at the time of diagnosis. Tumor cells invading the epidermis can be seen directly overlying the dermal component but these should not extend beyond the width of three rete ridges in any section (Clark et al, 1969).

Lentigo maligna melanoma
LMM arises on sun-damaged skin of elderly patients and are associated with lentigo maligna which is an in situ lesion characterized by profound lentiginous proliferation of atypical melanocytes in the vicinity of the tumor and in hair follicles. They are considered the least malignant melanomas as the in situ phase is often so prolonged.

Acral lentiginous melanoma
ALM arises on palmar and plantar skin along with the nails. This subtype is uncommon in Caucasians but the most common type found in Orientals and black people.

Other rare subtypes are also known such as desmoplastic melanoma that can be difficult to diagnose as this type is made of spindle cells with substantial fibrosis. This tumor type has a tendency to infiltrate around nerves and commonly develops local recurrences, however it rarely metastasizes.
Histopathology

The micoscopical features of malignant melanoma tumors vary widely. The cells can be large and rich in cytoplasm, small or even spindly. In some tumors there are areas with abundant melanin but in others the pigment is not so obvious. The nuclei are enlarged, often with a prominent nucleolus and mitoses are seen although the number varies. The microscopical appearance can also vary between different areas in the individual tumor (Fig. 4).

When the diagnosis of a malignant melanoma is made certain histopathological factors apart from the subtype should be reported:

The thickness of the tumor

The thickness of the tumor is the most important histopathological factor as it is an independent prognostic factor (Breslow, 1970). The thickness divides the tumors into different T-stages (Table 1 and 2). The thickness is measured in mm (Breslow) from the stratum granulosum of the epidermis to the deepest tumor nest.

Ulceration

The presence of ulceration is evaluated in the microscope and means that the epidermis is eroded. Ulceration is a prognostic factor (Balch et al, 1980; McGovern et al, 1982) included in the staging of the tumor (Table 1 and 2). Ulceration probably reflects rapid tumor growth.

Mitotic rate

Recently the TNM (tumor, lymph node, metastasis) (Table 1 and 2) classification for malignant melanomas was revised and a new prognostic factor, mitotic rate, was included for defining T1 tumors (Balch et al, 2009a; Balch et al, 2009b; Gershenwald et al, 2010; Nading et al, 2010). Multivariate analysis on thousands of patients with malignant melanomas including T-stage, ulceration and mitotic rate revealed that Clark levels (see below) gave non-significant survival results and therefore Clark levels are no longer used for defining T1 melanomas.
Table 1: TNM staging categories for cutaneous malignant melanoma (Balch et al, 2009a; Balch et al, 2009b).

<table>
<thead>
<tr>
<th>Primary Tumor</th>
<th>Thickness (mm)</th>
<th>Ulceration/Mitoses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tis</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>T1</td>
<td>≤1</td>
<td>a. Without ulceration and mitosis &lt;1/mm² b. with ulceration or mitoses ≥1/mm²</td>
</tr>
<tr>
<td>T2</td>
<td>1.01-2.0</td>
<td>a. without ulceration b. with ulceration</td>
</tr>
<tr>
<td>T3</td>
<td>2.01-4.0</td>
<td>a. without ulceration b. with ulceration</td>
</tr>
<tr>
<td>T4</td>
<td>&gt;4</td>
<td>a. without ulceration b. with ulceration</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regional Lymph Nodes</th>
<th>No. of Metastatic Nodes</th>
<th>Nodal Metastatic Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>1</td>
<td>a. Micrometastasis* b. Macrometastasis#</td>
</tr>
<tr>
<td>N2</td>
<td>2-3</td>
<td>a. Micrometastasis* b. Macrometastasis# c. In transit met(s)/ satellite(s) without metastatic nodes</td>
</tr>
<tr>
<td>N3</td>
<td>≥4, or matted nodes, or in transit met(s)/ satellite(s) with metastatic node(s)</td>
<td></td>
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<table>
<thead>
<tr>
<th>Distant Metastasis</th>
<th>Site</th>
<th>Serum LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastases</td>
<td>NA</td>
</tr>
<tr>
<td>M1a</td>
<td>Distant skin, subcutaneous or nodal metastases</td>
<td>Normal</td>
</tr>
<tr>
<td>M1b</td>
<td>Lung metastases</td>
<td>Normal</td>
</tr>
<tr>
<td>M1c</td>
<td>All other visceral metastases</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Any distant metastasis</td>
<td>Elevated</td>
</tr>
</tbody>
</table>

NA: not applicable, LDH: lactate dehydrogenase.
*Micrometastases are diagnosed after sentinel lymph node biopsy.
#Macrometastases are defined as clinically detectable nodal metastases confirmed pathologically.
Table 2: Anatomic stage groupings for cutaneous malignant melanoma (Balch et al, 2009a; Balch et al, 2009b).

<table>
<thead>
<tr>
<th>Clinical Staging*</th>
<th>Pathologic Staging#</th>
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<tbody>
<tr>
<td><strong>T</strong></td>
<td><strong>N</strong></td>
</tr>
<tr>
<td>0</td>
<td>Tis</td>
</tr>
<tr>
<td>IA</td>
<td>T1a</td>
</tr>
<tr>
<td>IB</td>
<td>T1b</td>
</tr>
<tr>
<td></td>
<td>T2a</td>
</tr>
<tr>
<td>IIA</td>
<td>T2b</td>
</tr>
<tr>
<td></td>
<td>T3a</td>
</tr>
<tr>
<td>IIB</td>
<td>T3b</td>
</tr>
<tr>
<td></td>
<td>T4a</td>
</tr>
<tr>
<td>IIC</td>
<td>T4b</td>
</tr>
<tr>
<td>III</td>
<td>Any T</td>
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<td></td>
<td></td>
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<td>IV</td>
<td>Any T</td>
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*Clinical staging includes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases.

#Pathologic staging includes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial (i.e. sentinel node biopsy) or complete lymphadenectomy. Pathologic stage 0 or stage IA patients are the exception; they do not require pathologic evaluation of their lymph nodes.

Clark level

The Clark levels (Clark et al, 1969) are divided into five groups depending on how deep a tumor infiltrates in relation to histological landmarks in the skin. Clark levels were previously used for defining T1 tumors (Balch et al, 2001a).

- **Clark I**: The tumor cells are confined within the epidermis, i.e. *in situ*
- **Clark II**: The tumor cells infiltrate the papillary dermis but are not filling out the whole papillary dermis
- **Clark III**: The tumor is filling out the papillary dermis but is not infiltrating the reticular dermis
- **Clark IV**: The tumor infiltrates the reticular dermis
- **Clark V**: The tumor infiltrates subcutaneous fat

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Other factors
Occurrence of perineural or intravascular growth should be noted and the resections margins evaluated by measuring the shortest margin.

Treatment
The primary treatment for malignant melanoma is wide local excision. For tumors ≤1mm in thickness, an excision with a 1 cm margin is satisfactory but for tumors >1mm an excision with a 2cm margin is recommended in Sweden (Ball & Thomas, 1995; Cohn-Cedermark et al, 2000). Subcutaneous tissue down to the fascia should be included. For patients with T2-T4 tumors sentinel lymph node mapping is recommended for patients with clinically uninvolved nodes as part of the staging process (Balch et al, 2009a; Balch et al, 2009b; Dessureault et al, 2001) (Table 1 and 2). For patients with T1b tumors sentinel lymph node mapping should also be considered. If positive, it is recommended to excise the regional lymph nodes as this prolongs disease free survival (Morton et al, 2006).

For an advanced disease there are no treatment options yet available which cure the patient (Garbe et al, 2010). For metastases located in the skin and subcutaneous tissue radical surgery is the best treatment option. As mentioned above if a sentinel node is positive the corresponding regional lymph nodes should be excised. The regional lymph nodes should also be removed if they are clinically involved at diagnosis (Morton et al, 1991). Surgery for distant metastases can be an option, particularly if the metastasis is solitary and accessible. Malignant melanomas are resistant to radiotherapy however radiotherapy can be applied if the resection of lymph node metastases is non-radical (Burmeister et al, 2006) or as a palliation for metastases in the brain or skeleton (Douglas & Margolin, 2002; Kirova et al, 1999; Rate et al, 1988). The effect of adjuvant chemotherapy for advanced disease is debated but meta-analysis have demonstrated better relapse free survival if interferon-α is used (Eggermont, 2001; Garbe & Eigentler, 2007). For palliation dacarbazin (DTIC) is primarily used but other drugs like interferon and interleukins-2 can also be tried (Garbe et al, 2010).

Prognosis
The prognosis for patients diagnosed with malignant melanoma has improved in Sweden. The greatest improvement in 5-year relative survival was seen in the period 1964-1993 where the relative survival rate improved for men from 58% to 84% and for females from 76% to 90%. However, during recent decades, a slight improvement was seen in the period 1994-2003.
where the relative survival rate for men improved from 84% to 86% and for females from 90% to 92% (Tryggvadottir et al, 2010).

The disease stage is the most important prognostic factor, which underlines the importance of early diagnosis. In Sweden, the 5-year melanoma specific survival rate for patients diagnosed between 1990-2005 was 91% for disease stage I-II, 37% for stage III and 24% for stage IV (Nationellt kvalitetsregister för melanom, 1990-2005). For patients diagnosed with a localized disease the main prognostic indicator is the T-stage. The 5-year melanoma specific survival rate in the period 1990-2005 for disease stage I-II was 98% for T1 tumors, 91% for T2, 76% for T3 and 62% for T4 tumors (Nationellt kvalitetsregister för melanom, 1990-2005). Other important prognostic factors indicating worse prognosis are increased mitotic rate and ulceration and as discussed previously these factors are included in the staging of a tumor. Other factors coupled to worse prognosis are higher age, male gender and a primary tumor located on the trunk (Balch et al, 2009b; Balch et al, 2001b).

To date, the only serum biomarker included in the TNM staging is measurement of lactate dehydrogenase (LDH) for patients in stage IV (Table 1) (Balch et al, 2009b; Deichmann et al, 1999; Eton et al, 1998; Sirott et al, 1993). LDH is elevated in other tumor types and indicates a high tumor load.

Prevention
To prevent malignant melanoma tumors from developing it is important for the public to be aware of the harmful effects of the sun and behave accordingly. Use clothing appropriately and in particularly protect the children. When the tumor has arisen it is important to detect it at an early stage and therefore both the public and health care personal need to recognize the alarming macroscopical features of a tumor. In addition it must be easy for the public to seek help.

Immunohistochemistry
Because of the varied microscopical appearance of malignant melanoma tumors IHC is often used to distinguish malignant melanoma from other tumor forms. Traditionally S-100 (Nakajima et al, 1982) has been used as an immunohistochemical marker of melanocytes but this protein also stains positive in e.g. Langerhans cells and nerve fibers. Other markers like Melan-A (MART-1) (Kawakami et al, 1994), HMB45 (Ordonez et al, 1988) and tyrosinase (Hofbauer et al, 1998) stain melanocytes more specifically but because they lack the sensitivity of S100 a combination of S100 with melanocytic markers is often used in clinical pathology (Ohsie et al, 2008). In
addition to markers of differentiation, proliferation markers are also widely used in the differential diagnostics of melanocytic lesions with uncertain malignant potential. The most accepted markers for cells active in the cell cycle are antibodies binding to Ki-67 and frequency of Ki-67 positive melanocytic cells is often used in clinical pathology to distinguish a malignant lesion from benign variants (Gerdes et al, 1984).

**Signal pathways**

The RAS-RAF-MEK-ERK-MAP kinase pathway that facilitates signal transduction in response to growth stimuli has mutated components in the majority of malignant melanoma cases studied. Activation of this pathway affects in the end proliferation, survival and invasion. RAS has three mutated oncogenes called KRAS, NRAS and HRAS, the result of different point mutations, but the NRAS mutation encompassing codon 61 is the most common one in primary sporadic malignant melanoma (Platz et al, 2008). Other forms of mutated RAS are more common in other types of cancer (Bos, 1989). RAF constitutes three closely related proteins called ARAF, BRAF and CRAF but mutations in BRAF were identified in 66% of uncultured melanomas (Davies et al, 2002) but subsequent studies have shown a range of 20-80% (Platz et al, 2008). The most common mutation is in codon 600 (Kumar et al, 2003).

Although most malignant melanomas are sporadic, familial forms occur with mutations in the cyclin-dependent kinase inhibitor 2A (CDKN2A) gene in the affected families. There is a variation in the mutation frequency reported (20-57%), partly because of a geographic variation (Goldstein et al, 2007; Hayward, 2003). The gene produces two interrelated proteins, p16/INK4a and p14/ARF, depending on the promoter used but both are involved in cell cycle regulation. p16/INK4a affects phosphorylation of the retinoblastoma protein that normally functions as a cell-cycle regulator but p14 affects the p53 tumor suppressor pathway.

**Potential biomarkers**

Numerous studies based on IHC have been published describing the protein expression of various potential biomarkers in malignant melanomas. The proteins studied have various functions (reviewed by Bosserhoff, 2006; Gould Rothberg et al, 2009b; Utikal et al, 2007) and many have shown promising results but none has hitherto been able to substitute the previously discussed established prognostic factors and therefore they have not been implemented into clinical practice. For a promising marker it is important to do a multivariate analysis to test if this marker is independent of T-stage as it
is only such markers that are of interest. For those who are not independent of T-stage, the T-stage remains a better prognostic marker. Regarding the prognostic significance of Ki-67 results have been confounding with some studies revealing a prognostic significance independent of tumor thickness (Gimotty et al, 2005; Ramsay et al, 1995) but other studies have not concluded so (Ilmonen et al, 2005; Ohsie et al, 2008). Other studies, also based on IHC have focused on comparing the expression of proteins during progression of the disease. In one of these studies different expression patterns of various proteins were found depending on the stage of the progression (Alonso et al, 2004).

The thickness is still the most important prognostic factor, however the thickness alone does not identify the patients that are at increased risk of dying from their disease despite a T1 or T2 tumor. Those patients might have a certain prognostic protein panel combined of e.g. 3-5 proteins, which can be employed to detect differences at protein level and place patients into certain risk groups.
Proteins studied in the thesis

The HPA project is a valuable resource for identifying potential biomarkers. However, their original screening on 12 melanoma cases warrants further evaluation in larger patient cohorts with clinical data if the aim is to identify a prognostic marker. For a promising marker it is important to verify the results in more than one cohort and automated analysis (see below) is also a valuable tool for confirming manual results.

The proteins included in this thesis were selected as they had an interesting staining pattern in the original HPA project screening. Some were selectively expressed in the malignant melanoma tumors but others were also expressed in other tumor types. The expression pattern varied with some proteins highly expressed in the majority of the 12 cases but others were more differentially expressed. Part of the proteins had previously been described in malignant melanoma tumors but generally limited knowledge was available coupling their protein expression to survival.

Examples of proteins studied:

Syntaxin-7 (STX7)

In humans 15 different syntaxin proteins are known. They are important for the intracellular trafficking, participating in the formation of transport vesicles in the exocytotic pathways and endocytotic pathway where lysosomes are the final compartment (Teng et al, 2001). Syntaxin proteins belong to the SNARE protein family (soluble N-ethylmaleimide-sensitive factor-attachment protein receptors). STX7 (Wong et al, 1998) is involved, as the other syntaxins, in the intracellular vesicular trafficking but the exact location is unknown. Articles have been published describing STX7 in early endosomes (Prekeris et al, 1999), in late endosomes (Nakamura et al, 2000), in late endosomes and lysosomes (Mullock et al, 2000) and to play a role in phagocytosis (Collins et al, 2002). The role of STX7 in melanocytes is unknown.
SRY (sex determining region Y)-box 10 (SOX10)

Neural crest cells are found early in the developing embryo and they give rise to cells with migratory potential that have very different functions. The cells derived from the neural crest multipotent cells include neurons and glia cells of the peripheral nerve systems, melanocytes of the skin and cartilage and bone of the face (Huang & Saint-Jeannet, 2004). Sox proteins are a group of transcription factors that are widely found in the animal kingdom. In mammals 20 different genes are known (Harris et al; Schepers et al, 2002). Sox10 is believed to be essential for neural crest cell fate determination (Kim et al, 2003; Kuhlbrodt et al, 1998) and to maintain the multipotency of neural crest cells (Kelsh, 2006). Recently IHC has been employed to map the expression of SOX10 in various human tissues and SOX10 has been suggested to be a more specific and sensitive marker for melanocytic tumors than S100 (Nonaka et al, 2008; Ohsie et al, 2008) and also to be a reliable marker for the detection of melanoma cells in sentinel lymph nodes (Blochin & Nonaka, 2009). SOX10 immunostaining has also been proposed a good marker for differentiating desmoplastic melanoma from scar tissue (Ramos-Herberth et al, 2010).

Microphthalmia associated transcription factor (MITF)

This protein is important for the differentiation and survival of melanocytes in addition to the production of melanin pigment (reviewed by Levy et al, 2006; Steingrimsson et al, 2004). The MITF protein is a basic helix-loop-helix leucine zipper (b-HLH-Zip) transcription factor (Hodgkinson et al, 1993) where nine different isoforms are known in humans. The different isoforms are the results of nine different promoters within the MITF gene. The MITF-M isoform is melanocyte specific (Fuse et al, 1996). SOX10 protein, along with PAX3 regulate the promoter of the MITF gene (Lee et al, 2000; Verastegui et al, 2000). One study has indicated that amplification of the MITF gene is associated with progression of disease and risk of distant metastases (Garraway et al, 2005) but other clinical studies have shown that a high expression on the contrary may be beneficial (Salti et al, 2000). In vitro and animal studies implicate a complex pattern where both depletion and forced expression inhibit proliferation in cell lines (Kido et al, 2009) and high levels of MITF inhibit tumor growth and decrease Ki-67 expression (Lekmine et al, 2007). Studies focusing on MITF and IHC have revealed that MITF antibodies are not suitable to employ when diagnosing melanocytic lesions as they are not specific or sensitive enough (Busam et al, 2001; King et al, 2001; Miettinen et al, 2001). Despite that further research into the role of MITF is of interest since it might be a potential therapeutic target.
RNA binding motif 3 (RBM3)

RNA binding proteins are important for RNA metabolism and gene transcription. Different groups exist depending on the RNA binding motif of the particular protein (Burd & Dreyfuss, 1994). One group of proteins, which contain 1-4 copies of a RNA recognition motif (RRM), are called RNA binding motif (RBM) proteins and RBM3 belongs to this group (Sutherland et al., 2005). RBM3 was first described in 1995 (Derry et al., 1995) but still the exact function is unknown. In one study (Baldi et al., 2003) a cDNA array was employed to detect down-regulation of the RBM3 gene during progression of malignant melanoma. This research was based on two melanoma cell lines from the same patient where one cell line was derived from the primary tumor and the other from a metastasis. Recently, RBM3 positivity has been identified as a good prognostic marker in breast cancer (Jogi et al., 2009) and ovarian cancer (epithelial type) (Ehlen et al., 2010).
In this thesis the manual annotation of the IHC staining was reported in regard to the fraction of positive tumor cells focusing on the cytoplasm or nucleus depending on the localization of the particular protein. For most of the proteins studied the fraction was divided into four groups: >75% of the tumor cells staining positively, 25-75% staining positively, <25% and a negative staining. In addition the intensity was evaluated on a three-graded scale: a strong staining, a weak staining and negative. For the proliferation marker Ki-67 the tumors were divided into two groups, <20% positive cells and ≥20% (Alonso et al, 2004; Hazan et al, 2002; Ramsay et al, 1995). For several proteins which were located in the cell membrane human epidermal growth factor receptor 2 (HER-2) annotation was employed (Jacobs et al, 1999): negative or membranous positivity in <10% of the tumor cells, a weak incomplete membranous positivity in >10% of the tumor cells, a weak to moderate complete membranous positivity in >10% of the tumors cells, a strong complete membranous positivity in >10% of the tumor cells. For one protein, RBM3, the fraction of positive tumor cells was evaluated in the following intervals: 0-1%, 2-10%, 11-25%, 26-50%, 51-75%, >75%. The intensity of the staining was evaluated as strong, moderate, weak or negative. The different annotation strategies are in part related to increased knowledge and changed strategies within the HPA project through the years this research was conducted. Evaluating the majority of proteins in only four groups in terms of fraction might some think as crude but it can also be argued that a very good biomarker is either on or off.

It is important to be aware of that a manual annotation is a subjective evaluation and a very time consuming task when working with multiple samples. For malignant melanoma the annotation is also difficult as the tumor cylinders are relatively small, in some tumors there is a lot of melanin and in others the tumor is not represented and therefore it is important to be able to recognize tumor from normal tissues.

One way of verifying manual results is to employ a complementary automated image analysis system (Mulrane et al, 2008; Rexhepaj et al, 2008). An automated analysis relies on a computer algorithm that recognizes the color produced with the IHC staining procedure. The particular algorithm is able to detect the difference between tumor and stroma in order to evaluate the correct component. With the automated annotation more variation in
color intensities (on a continuous scale) can be detected compared with the human eye, which has a limited capacity to do so. In addition, the problem of inter- and intra-observer variability is avoided but automated algorithms are increasingly used for evaluation of IHC (Cregger et al., 2006; Decaestecker et al., 2009; Gould Rothberg et al., 2009a).

Rimm and co-workers have developed an automated quantitative analysis (AQUA) method, which is based on IF. In this automated algorithm a protein specific to the tumor being studied is used to map the tumor area and then within this area IF is used to detect and quantify the antigen of interest and the subcellular area (Camp et al., 2002). They have previously described individual prognostic markers in malignant melanoma using this method (Berger et al., 2004; Divito et al., 2004) and recently they published an article with promising prognostic results for a panel of five markers (Gould Rothberg et al., 2009a). The markers included are: ATF2, p21\(^{WAF1}\), p16\(^{INK4A}\), \(\beta\)-catenin and fibronectin.
Present investigation

Aims of the present investigation:

- To identify potential biomarkers in malignant melanoma
- To study the expression of selected candidate biomarkers in defined patient cohorts employing tissue microarrays and immunohistochemistry
- To test and compare manual and automated methods for analysis of immunohistochemistry-based protein expression
- To investigate/analyze possible correlations between protein expression profiles of identified candidate biomarkers and clinical parameters including survival
Summary of results and discussion

This thesis is based on four papers where focus lies on identifying potential new prognostic markers in malignant melanoma. This chapter summarizes the results and discussion for each paper, followed by conclusions and future perspectives.

Paper I

Selective expression of syntaxin-7 protein in benign melanocytes and malignant melanoma

In the first paper the protein expression of STX7 was extensively studied employing TMAs and IHC. STX7 is a protein that is located in the cytoplasm and participates in vesicular trafficking, although the exact location is unknown. This protein was identified as a novel protein strongly expressed in many cases of malignant melanomas in the HPA screening process and in lymphomas. Subsequently the protein was characterized in regard to expression pattern in melanocytes in normal skin, benign nevi and two malignant melanoma patient cohorts. In addition, the subcellular localization and the expression pattern in different cell lines was determined and the protein’s role as a prognostic marker was studied. The tissue material was composed of 18 normal skin samples, 12 different benign melanocytic lesions and two different patient cohorts where the patients had been diagnosed with infiltrative cutaneous malignant melanoma (cohort I: 151 patients and 35 metastasis, cohort II: 165 patients). Various clinical parameters were available but survival information was only available for cohort I.

STX7 was highly expressed in cells of the melanocytic lineage. For the malignant tumors STX7 expression was inversely correlated with T-stage (Spearman’s Rho = -0.28; p=0.001) and was even stronger expressed in SSMs compared with NMMs (p=0.009) in cohort II. However, no correlation with overall or disease-free survival was observed. The protein was expressed in two melanoma cell lines but not in two epithelial cell lines. As for the subcellular localization the protein was visible in the cytoplasm as would be expected but the protein was also identified in the nucleus, which can indicate an unknown role of the protein.
This study describes for the first time the expression of STX7 in cells of the melanocytic lineage and illustrates an approach to identify potential clinical biomarkers with antibody-based proteomics.

Paper II

SOX10 expression in superficial spreading and nodular malignant melanomas

In the second paper the protein expression of SOX10 was determined in 106 primary tumors (SSM and NMM) and 39 metastases in addition to 16 normal skin samples and six benign nevi employing IHC and TMAs. SOX10 is a transcription factor important for the differentiation of melanocytes and for neural crest cells, both to maintain their multipotency and for the differentiation of neural crest derived cells. This protein has been suggested to be a more specific marker of melanocytes than S100. As for the IHC staining it was evaluated both manually and with an automated algorithm. In addition, the effect of SOX10 on migration and proliferation was analyzed extensively in vitro employing SOX10 small interfering (siRNA) mediated silencing in three different melanoma cell lines.

SOX10 was strongly expressed in the benign melanocytic tissues but for the malignant tumors SSMs stained stronger compared with NMMs (p=0.008), the weakest staining was observed in lymph node metastases (automated results). SOX10 staining intensity was inversely correlated with T-stage (Spearman’s Rho = -0.261; p=0.008) and the proliferation marker Ki-67 (Spearman’s Rho = -0.173; p=0.02, automated results only). In univariate analysis SOX10 intensity was significantly correlated with overall survival and time to recurrence but in multivariate analysis including T-stage the results were non-significant. SOX10 down-regulation resulted in variable effects on proliferation and migration rates in the cell lines and therefore no firm conclusions could be drawn in regard to the role of SOX10 for proliferation and migration.

This study describes the expression of this particular protein in different melanocytic lesions. The protein was expressed in all the primary tumors, which could indicate a role for this protein in malignant melanoma diagnosis. However, there were different intensity levels depending on the type of tumor. The intensity of the staining was also inversely correlated with T-stage and Ki-67. Although the functional studies could not indicate a firm role for SOX10 this demonstrates that cellular properties between cell lines can vary and possibly also between individual tumors although this remains to be established.
Paper III

MITF as a prognostic marker in cutaneous malignant melanoma

In the third paper a population-based cohort was employed to explore whether the protein expression of MITF was useful as a prognostic marker in patients operated on for malignant melanoma. MITF is a transcription factor important for the differentiation and survival of melanocytes, in addition to melanin production. A representative sample was drawn from patient-based registers with baseline characteristics available including survival information. TMAs were constructed and they included tissue material from 264 patients, including 45 patients that had died of malignant melanoma. With IHC the protein expression was studied. Looking at cell fraction and staining intensity separately patients with cell fraction >75% and patients with strong staining tumors had a lower risk of dying from malignant melanomas compared with the other groups. When fraction and intensity was combined a high-risk group dying from malignant melanoma was identified as those patients with 25-75% of the tumor cells staining with weak intensity or less than 25% of the tumor cells staining with strong intensity. However, as the high-risk expression included less than 15% of all patients and identified only 12 of 45 deaths in the cohort it was concluded that MITF was not a favorable biomarker on its own. Perhaps, if combined with other markers MITF might have a prognostic role. In this paper the expression of a particular protein is described in a representative sample from a whole population and although this marker was not useful as a prognostic marker the results are in line with experimental studies and are relevant to explore further as MITF might be an interesting therapeutic target.

Paper IV

Protein biomarkers in malignant melanoma: An image analysis-based study on melanoma markers of potential clinical relevance

In the fourth paper results for 21 proteins studied in malignant melanomas were presented. As in the other papers the tumors were in a TMA format and IHC was employed. The expression was studied in 143 tumors (SSM=96, NMM=47). The aim was to identify novel prognostic and diagnostic melanoma biomarkers. For most of included markers the IHC staining was evaluated manually by looking at the fraction of positive cells and the staining intensity. MITF (Rho=0.477, p<0.001) and STX7 (Rho=0.395, p<0.001) correlated best with the expression of the established melanocyte marker Melan-A as has been described previously. For all the protein markers disease free survival was computed and in univariate analysis significant results
were seen for RBM3, SOX10 and Ki-67. However, in multivariate analysis including T-stage non-significant results were seen. When looking at MITF intensity values and disease free survival two separate groups were seen, although non-significant. To explore the manual results more thoroughly a hierarchical clustering approach was used to generate a dendogram to identify markers with the highest variance (entropy) driving hierarchical grouping. This analysis identified the proteins RBM3 and Ki-67. The combination of RBM3, SOX10, MITF and Ki-67 expression data was tested with an algorithmic approach and resulted in a protein signature that was independent of T-stage in multivariate analysis ($p=0.009, HR=0.45$ (95% CI 0.25-0.82)). Good prognosis was coupled to many tumor cells staining for RBM3, weak SOX10 and MITF staining intensity and few cells staining for Ki-67.

Five additional proteins (ATF2, $p21^{WAF1}$, $p16^{INK4A}$, $\beta$-catenin and fibronectin) were included in an automated analysis based on IHC. The combination of these particular proteins has previously been identified to constitute a prognostic panel employing an automated approach based on IF. With the automated algorithm based on IHC a trend towards significant disease free results were seen ($p=0.09$).

The thickness of a primary malignant melanoma tumor remains the most important prognostic marker for the individual patient with a localized disease. However, it cannot on its own sufficiently identify the patients that risk death from advanced malignant melanoma. Therefore it is important to look for other characteristics that have a prognostic value. This is the focus of the last paper and although our results are promising we are still working on them and they also need to be confirmed in other patient cohorts.

Conclusions and future perspectives

In this thesis the focus was to study the protein expression of various proteins in cutaneous malignant melanoma and for some of the proteins the expression was also studied in melanocytes found in normal skin and in benign nevi. The protein expression was studied with IHC in multiple tumors arranged in a TMA format and several cohorts were employed to identify novel prognostic and diagnostic melanoma biomarkers. The antibodies were validated within the HPA project and the staining procedure standardized as much as possible and therefore we believe our results can be relied upon.

The evaluation of the IHC staining was done manually and for part of the proteins also with an automated algorithm. Manual evaluation is a time consuming task, which can be avoided if automated algorithms are used. With automated algorithms the color signal produced with the staining procedure is evaluated on a continuous scale. This allows for the detection of important differences, which can be missed when the evaluation is done manually as
the human eye has a limited capacity to detect differences in color intensities.

The main conclusions that can be drawn from this work are that we have extensively studied the protein expression of different protein. A few of the studied proteins have not been characterized previously in malignant melanomas (e.g. STX7, RBM3). For some proteins we have seen differences in intensities depending on the subtype (STX7, SOX10) where SSMs stained stronger compared with NMMs. As these tumor forms look different in the light microscope it’s not surprising to identify markers that demonstrate different expression patterns with underlying molecular events that differ. An inverse correlation between T-stage and the fraction of positive STX7 tumor cells was seen and an inverse correlation was also seen between T-stage and the SOX10 staining intensity. How to explain these correlations is however, not clear. We found a significant correlation with survival in univariate analysis for SOX10 intensity although not significant in multivariate analysis including T-stage, which demonstrates how important the T-stage is a prognostic factor. Other proteins (e.g. STX7, MITF) demonstrated a good correlation with Melan-A expression data. Although in reality Melan-A, tyrosinase and HBM45 are very specific melanocyte markers there is still a need to identify additional markers may be of value for better detection of rare malignant melanoma subtypes, like desmoplastic melanoma and for understanding melanocyte/melanoma biology.

Different cohorts were analyzed to study the results in regard to survival and in one of the studies a population-based cohort was employed to study the distribution of MITF expression in the population and in that way decide whether MITF could be used as a prognostic marker. A high-risk group dying of malignant melanoma was identified but because the distribution of these particular expression patterns was limited in the population and identified few deaths it was concluded that MITF was not useful as a prognostic marker on its own.

Extensive migration and proliferation studies were performed in one of the papers employing siRNA that targeted SOX10. The results were different in the three cell lines studied and therefore no firm results regarding the role of SOX10 on these factors could be drawn. Functional studies like these are important to better understand the specific role of a particular protein.

We have employed an automated image analysis system to confirm our manual annotations and we have used bioinformatics tools to explore more thoroughly our manual results in order to detect interesting combination of markers (a protein signature) in regard to survival. We identified a protein signature, including four proteins: RBM3, SOX10, MITF and Ki-67, which gave significant survival results in both univariate and multivariate analysis including T-stage for disease free survival. RBM3 is a novel protein not previously characterized in malignant melanomas and this protein was driv-
ing the signature. These results are very interesting and need to be confirmed in other cohorts also.

In this era of molecular research it’s fascinating that no molecular factor has been able to replace the thickness of a primary malignant melanoma tumor as a prognostic marker. However, molecular research has the potential to identify subtle differences between tumors and in that way identify patient groups that are at high-risk of dying from their disease. These patient groups might benefit from additional adjuvant therapy in the future or more frequent controls. Malignant melanomas are tumors with an ever increasing incidence the last few decades in the developed countries and therefore it is important to investigate this tumor form thoroughly to better understand its behavior and to better target the tumors when they arise with new therapeutic drugs. The incidence of malignant melanoma will hopefully diminish in the future as people become more aware of the harmful effects of ultraviolet radiation and protect themselves and their children accordingly. With increased awareness of this tumor form they are now diagnosed at an earlier stage and compared with other tumors located in the visceral organs they are relatively easy to identify and diagnose.

I believe this work has shed light on the molecular events, which are important for malignant melanoma tumors and perhaps some of the proteins, which we have studied will be of importance in the clinical work in the future. We have demonstrated a strategy, which can be employed when working with multiple protein markers with the aim of identifying new potential biomarkers.
Malignt melanom är en typ av hudcancer som härstammar från celler som kallas melanocyter som finns spridda i huden. Deras roll är att skydda keratinocyterna som bildar vårt yttersta hudlager från skada orsakad av ultravioletta solstrålarna. Denna hudtumör är mycket malign i sin natur, speciellt om den har hunnit sprida sig till lymfkörtlar eller andra organ. De senaste årtionden har antalet patienter som får diagnosen malignt melanom ökat drastiskt. Orsaken till denna ökning är delvis okänd men har delvis koppling till ändrade solvanor. För den enskilde patienten som inte har tecken på spridd sjukdom finns några prognostiska faktorer som kan bedömas vid ljusmikroskopisk undersökning av den bortopererade lesionen. Den absolut viktigaste faktorn är tumörens tjocklek mätt i mm men tjocka tumörer har större chans att sprida sig jämfört med tunna tumörer. Trots ökad kunskap om olika proteiner och gener som är viktiga i denna tumör har inget protein hittills visat sig vara bättre prognostisk markör än tjockleken.

Vi har studerat flera olika proteiner i bortopererade vävnadsprover från patienter med malignt melanom. Målet med studien var att hitta proteiner som kan hjälpa till vid att förutsäga patientens prognos. Vi har färgat för dessa proteiner på ett specifikt sätt som tillåter oss att bedöma hur mycket av varje protein melanomcellerna innehåller. Med statistiska analyser har vi fått fram eventuella kopplingar mellan proteinmängden och överlevnad samt olika kliniska faktorer.

Vi har sett att om man kombinerar resultat från fyra proteiner som vi har undersökt kan man förutsäga prognosen för patienterna. Denna index är oberoende av tumörens tjocklek och har således en prognostisk betydelse. Vi har också tittat på flera enskilda proteiner och beskrivit uttrycket för delvis okända proteiner och för vissa av proteinerarna har vi beskrivit uttrycket inte bara i tumörvävnad utan också i melanocyter i normal hud och i godartade födelsemärken (nevi). Proteinuttrycket har vi bedömt både med att titta på de färgade glasen i mikroskopet men också har vi använt en automatiserad datorteknik för att bekräfta våra manuella resultat.

Alla dessa studier bidrar till kunskapen om maligna melanom och det är vårt hopp att proteinuttrycksmönster kan i framtiden lättare identifiera patienter som riskerar död i malignt melanom trots en ganska tunn tumör från början.
Sortuæxlí er húðkrabbamein sem er myndað af sérhæfðum frumum sem kallast litfrumur (melanocytes). Þessar frumur eru undir eðlilegum kringum-stæðum dreifðar um húðyfirborda og mynda sérstakt litarefni, melanin, sem ver hornfrumur (keratinocytes) húðarinnar fyrir skaðlegum áhrifum út-fjórlublárrar geislunar. Síðustu áratugina hefur nýgengi sortuæxliis aukist mjög hjá vestreinum þjóðum og eru orsakir þessa að stórum hluta tengdar breytttri hegðun í söl. Hjá sjúklingi sem ekki er með merki um sjúkdóminn nema á einum stað í húðinni má dæma nokkra þættir sem segja fyrir um horfur sjúklingsins við smásjárskoðan af æxlinu þegar búið er að fjarlægja það. Aðrar rannsóknar hafa synt að mikilvægasti þátturinn við að meta horfur sérhvers sjúklings er þykkt æxliins í húðinni með í mm en meiri líkur eru á að þykktæxlí hafi dreifð sér til eftir og annarli líkur meliðið við þunnt æxli. Þrátt fyrir umfangsmiklar rannsóknar rannsóknir á bæði prótínum og genum er þykktin enn besti þátturinn til að meta horfur sjúklingsins.

Í þessari rannsókn höfum við rannsakað tjáningu fjöldra prótina í sortuæxlum þar sem tilgangurinn var að finna eitt prótín eða hóp prótína sem gætu spáð fyrir um horfur sjúklingsins og þannig greint t.d. vissan áhættuhóp hjá sjúklingum með þannæxli sem venjulega hafa mjög góðað horfur. Með því að líta fjölda vegjafræna frá sortuæxlum á sérstakan hátt var hægt að meta magn prótínum anna í æxlunum og þar að fj'algera tolfmæðilega greiningu á niðurstoðum til að finna út tengsl við horfur og einstaka sjúklingatengda þættir.

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Þessi rannsókn hefur aukið þekkingu okkar á sortuæxlum m.t.t. prótíntjánings en það er von okkar að tjáningarnunstur prótina geti í framtiðinni hjálpað til við að finna þá sjúklinga sem eru í aukinni hættu að að deyja úr sortuæxli.
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