

A high-throughput pathology approach for further insight into
the role of RBM3 as a biomarker of prognosis and
chemotherapy response in human cancer

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DOCTORAL DISSERTATION

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Title: A high-throughput pathology approach for further insight into the role of RBM3 as a biomarker of prognosis and chemotherapy response in human cancer		
Abstract: Cancer incidence is increasing and it is the number one cause of death worldwide. Cancer is a highly heterogeneous disease and there is a great need for new early diagnostic, prognostic and treatment predictive biomarkers in order to improve patient outcomes. The RNA-binding motif protein 3, RBM3, is an emerging candidate biomarker of favourable prognosis and treatment response in several types of human cancers. The aim of this thesis was to further investigate the expression, clinicopathological correlates, prognostic and predictive significance of RBM3 in malignant melanoma, prostate cancer, upper gastrointestinal cancer and pancreatic and periampullary cancer. In the latter, the expression of another RNA-binding protein and biomarker candidate, the Hu-antigen R, HuR, was also examined. All the analyses are based on immunohistochemistry (IHC), performed on tissue microarrays (TMA). RBM3 expression was examined in primary tumours and metastases from 215 patients with malignant melanoma (Paper I), in primary tumours and paired normal tissue from 88 patients with prostate cancer (Paper II), in primary tumours, metastases, normal tissue and cases of intestinal metaplasia (IM) from 173 patients with oesophageal and gastric adenocarcinomas (Paper III), and in primary tumours, metastases and normal tissue from 171 patients with pancreatic and periampullary adenocarcinomas (Paper IV). High RBM3 expression was associated with favourable clinicopathological characteristics and was found to be an independent factor of improved survival for patients with malignant melanoma, prostate cancer and oesophageal and gastric adenocarcinomas. RBM3 expression was lower in metastatic as compared with primary melanoma, similar in primary and metastatic oesophageal/gastric cancer, and higher in metastatic as compared with primary pancreatic/periampullary cancer. In pancreatic/periampullary cancer, high RBM3 expression was associated with unfavourable clinicopathological characteristics and was found to be an independent factor of poor prognosis in patients not receiving adjuvant chemotherapy. In contrast, in patients treated with adjuvant therapy, high RBM3 expression was an independent factor of improved survival, with a significant treatment interaction. Cytoplasmic HuR expression was significantly lower in metastatic as compared with pancreatic/periampullary cancer. High cytoplasmic HuR expression was associated with a prolonged survival in patients not receiving adjuvant treatment, but not prognostic in treated patients. Key words: RBM3, HuR, malignant melanoma, prostate cancer, upper gastrointestinal cancer, pancreatic and periampullary cancer, tissue microarrays, immunohistochemistry, chemotherapy		
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KLIMATKOMPENSERAT
PAPPER



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List of papers

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- I. **Jonsson L***, Bergman J*, Nodin B, Manjer J, Pontén F, Uhlén M, Jirstrom K. Low RBM3 protein expression correlates with tumour progression and poor prognosis in malignant melanoma: An analysis of 215 cases from the Malmö Diet and Cancer Study. *Journal of Translational Medicine* **9**:114 (2011)
- II. **Jonsson L**, Gaber A, Ulmert D, Uhlén M, Bjartell A, Jirstrom K. High RBM3 expression in prostate cancer independently predicts a reduced risk of biochemical recurrence and disease progression. *Diagnostic Pathology* **6**:91 (2011)
- III. **Jonsson L**, Hedner C, Gaber A, Korkocic D, Nodin B, Uhlén M, Eberhard J, Jirstrom K. High expression of RNA-binding motif protein 3 in esophageal and gastric adenocarcinoma correlates with intestinal metaplasia-associated tumours and independently predicts a reduced risk of recurrence and death. *Biomarker Research* **2**:11 (2014)
- IV. **Ben Dror L**, Elebro J, Gaber A, Heby M, Fristedt R, Olofsson SE, Nodin B, Uhlén M, Eberhard J, Jirstrom K. Expression of the RNA-binding proteins RBM3 and HuR in primary and metastatic pancreatic and periampullary adenocarcinoma: impact on prognosis and treatment response. *Manuscript*

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- Fridberg M, **Jonsson L**, Bergman J, Nodin B, Jirström K. Modifying effect of gender on the prognostic value of clinicopathological factors and Ki67 expression in melanoma: A population-based cohort study. *Biology of Sex Differences* **3**:16 (2012)
- Nodin B, Fridberg M, **Jonsson L**, Bergman J, Uhlén M, Jirström K. High MCM3 expression is an independent biomarker of poor prognosis and correlates with reduced RBM3 expression in a prospective cohort of malignant melanoma. *Diagnostic Pathology* **7**:82 (2012)
- Elebro J, Heby M, Gaber A, Nodin B, **Jonsson L**, Fristedt R, Uhlén M, Jirström K, Eberhard J. Prognostic and treatment predictive significance of SATB1 and SATB2 expression in pancreatic and periampullary adenocarcinoma. *Journal of Translational Medicine* **12**:289 (2014)
- Fristedt R, Elebro J, Gaber A, **Jonsson L**, Heby M, Yudina Y, Nodin B, Uhlén M, Eberhard J, Jirström K. Reduced expression of the polymeric immunoglobulin receptor in pancreatic and periampullary adenocarcinoma signifies tumour progression and poor prognosis. *PLOS ONE*, in press

Abbreviations

5-FU	5-flourouracil
AJCC	American Joint Committee on Cancer
ASCO	American Society of Clinical Oncology
ARE	AU-rich elements
BCR	Biochemical recurrence
BRAF	V-raf murine sarcoma viral oncogene homolog B
CA 19-9	Cancer antigen 19-9
CDKN-2	Cyclin-dependent kinase inhibitor 2
cDNA	Complementary DNA
CIRP	Cold inducible RNA-binding protein
COX-2	Cyclooxygenase 2
CRT	Classification and regression tree analysis
CSS	Cancer specific survival
dCK	Deoxycitidine kinase
DFS	Disease free survival
DSS	Disease specific survival
DNA	Deoxyribonucleic acid
CT	Computed tomographic

CTLA-4	Cytotoxic T-lymphocyte-associated antigen 4
ELAV	Embryonic lethal abnormal vision
ER	Oestrogen receptor
EGJ	Oesophagogastric junction
ERCP	Endoscopic retrograde cholangiopancreatogram
EUS	Endoscopic ultrasonography
FISH	Fluorescence in situ hybridization
GERD	Gastro-oesophageal reflux
GSTP-1	Glutathione-S-Transferase P1
HER-2	Human epidermal growth factor receptor 2
hnRNA	Heterogenous nuclear RNA
hnRNP	Heterogeneous nuclear ribonucleoprotein
HNS	HuR nucleocytoplasmic shuttling sequence
HPA	Human Protein Atlas
HuR	Hu-antigen R
IHC	Immunohistochemistry
IM	Intestinal metaplasia
IPMN	Intraductal papillary-mucinous neoplasm
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LHRH	Luteinizing hormone-releasing hormone
M0	Distant metastasis free disease
MAPK	Mitogen-activating protein kinase
MCM3	Minichromosome maintenance 3 protein

MCN	Mucinous cystic neoplasm
MDCS	Malmö Diet and Cancer Study
MGUS	Monoclonal gammopathy of unknown significance
MPE	Molecular Pathological Epidemiology
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
N-RAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
OR	Odds ratio
OS	Overall survival
PanIN	Pancreatic intraepithelial neoplasia
PET	Positron emission tomography
PFS	Progression free survival
PIN	Prostatic intraepithelial neoplasia
Pre-mRNA	Pre-messenger RNA
PrEST	Protein epitope signature tag
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
R0	Radically resected tumours
RBM3	RNA-binding motif protein 3
RBD	RNA-binding domain/motif
RBP	RNA binding proteins
RFS	Recurrence free survival
RNA	Ribonucleic acid

RRM	RNA recognition motif
RNP	Ribonucleoprotein
SCC	Squamous cell carcinoma
siRNA	Small interfering RNA
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonucleic particle
TMA	Tissue microarray
TNM	Tumour/Nodes/distant Metastases
tRNA	Transfer RNA
UTR	Untranslated region
WHO	World Health Organization

Introduction

Cancer is becoming an increasing problem in the world, partly as many people in developing countries adapt a “western” lifestyle with cancer-associated habits such as smoking, physical inactivity and change in diet, and also because of increasing age and growth of the world population. In economically developed countries, cancer is the primary cause of death and in economically developing countries it is the second leading cause of death. In 2012, an estimated 14.1 million new cases of cancer and 8.2 million cancer related deaths occurred worldwide and the most commonly diagnosed cancers are lung, breast and colorectal cancer, whereas the most common causes of death can be attributed to lung, liver and stomach cancer [1, 2]. In an effort to try to understand the mechanisms and complexity behind the development of cancer, Hanahan and Weinberg proposed six hallmarks of cancer in the year of 2000, all contributing to a multistep process by which the cell acquires tumorigenic characteristics and eventually the ability to spread. These steps include; self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and, lastly, tissue invasion and metastasis (Figure 1) [3]. Since the hallmarks of cancer were first proposed, considerable progress has been made in the understanding of carcinogenesis and a revised version was published in 2011 by the same authors. Among other things, two additional hallmarks were introduced, which contribute to the multistep process of carcinogenesis, i.e. genomic instability and cancer-related inflammation [4]. Other emerging hallmarks include reprogramming of energy expenditure and metabolism and also the capability of tumour cells to evade the immune system – both areas that need to be further investigated [4].

The Tumour/Nodes/distant Metastases (TNM) classification system, established by the American Joint Committee on Cancer (AJCC), is the most widely used system for staging of cancer, and the most important tool for prognostication and treatment stratification. The basis for the TNM system is anatomical and it is particularly useful when local treatment is the only cure, which was the case when it was introduced in the late 60’s [5, 6]. The manual is continuously being updated and revised, however, despite an immense need to incorporate additional biomarkers to further sharpen the tools for clinical decision making, this process has been very slow [5]. In an era where the human genome has been mapped, and

soon also the human proteome, and there is an exponential increase in scientific papers proposing novel or validating existing candidate biomarkers, this delay may seem unfathomable.

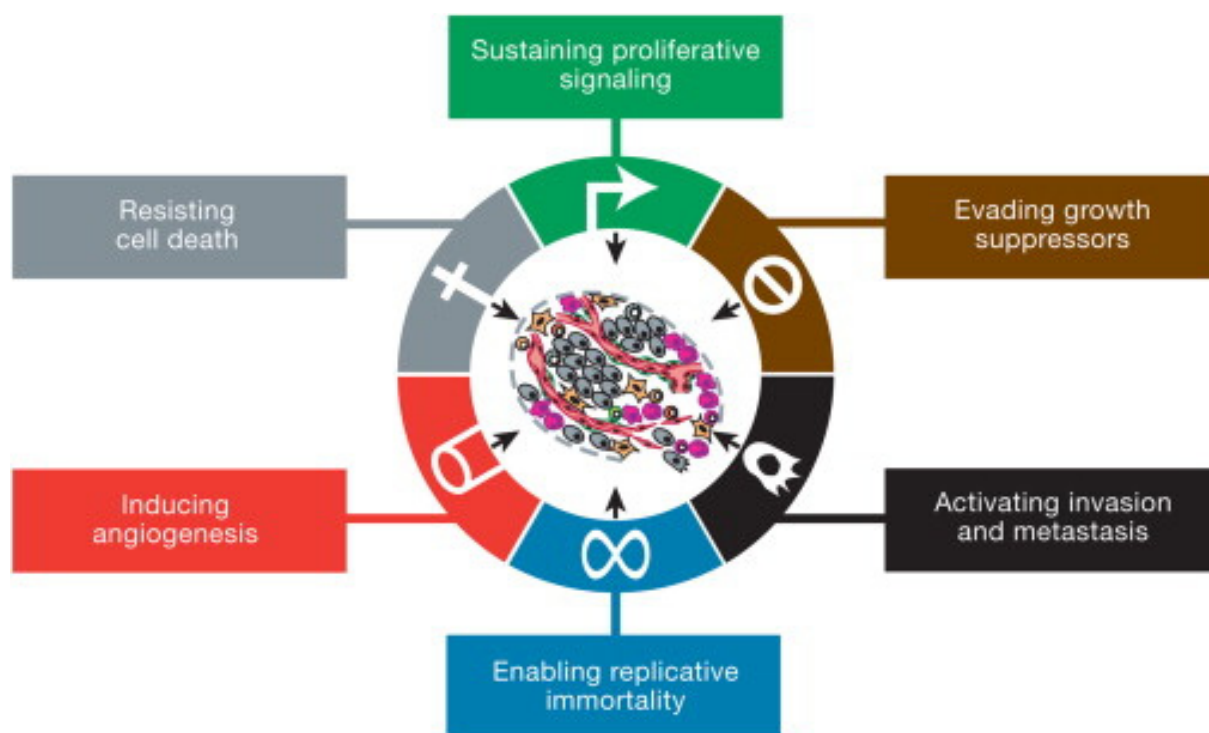


Figure 1. The six hallmarks of cancer. Reprinted with permission from Elsevier [4].

This thesis forms part of an ongoing effort to characterise the expression and clinicopathological correlates of the RNA-binding motif protein 3 (RBM3) in all major human forms of cancer, four of which are included herein. After completion of the first three papers, the notion of RBM3 being a nearly “universal” cancer biomarker of favourable prognosis seemed to be consolidated. However, in the last study, encompassing pancreatic and periampullary adenocarcinoma, the picture changed.

Antibody-based biomarker discovery

What is a biomarker?

The term biological marker, biomarker, is quite broad and some attempts have been made to define the term. In a joint project on chemical safety, “Biomarkers in risk assessment: validity and validation”, led by the World Health Organization (WHO) in collaboration with United Nations Environment Programme and the International Labour Organization, a biomarker was defined as “any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome of disease” (<http://www.inchem.org/documents/ehc/ehc/ehc222.htm>). The National Institute of Health defines a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention” [7].

Biomarkers are today an integrated part of medicine and encompass everything from blood pressure to measurements of elevated plasma troponin as an indicator of a cardiovascular event and they can be found for instance in blood, urine, tissues and sputum. Within research, two main areas of biomarker studies can be defined: One is the use of biomarkers for development of new drugs, mainly within the pharmaceutical industry, and the second one is the arena of studies related to disease mechanism, monitoring and prediction [8].

With the advent of various –omics technologies within the field of cancer research, there has in recent years been a great focus on finding new biomarkers, for screening and diagnosis as well as for monitoring of disease progression and prediction of therapeutic response or toxicity. Despite these advances, and a plethora of published biomarker papers, very few cancer biomarkers have been introduced into clinical practice, mainly due to problems with validation [9].

Immunohistochemistry

Immunohistochemistry (IHC), the use of colour-tagged antibodies in the search for cell or tissue antigens *in situ*, was initially introduced by Dr Albert Coons in the 1940's, who used fluorescein-labelled antibodies to localize antigens in tissue [10]. The technique has since then developed and is today used extensively within the field of histopathology and involves slide preparation as well as interpretation and quantification of the resulting expression patterns. In the process of preparing a slide, although being a rather simple technique, there are important factors to take into consideration to gain the best possible result. For instance, one needs to pay attention to the morphology of the section and the antigenicity of the component of interest during the fixation process. Other aspects to consider are the thickness of the slice when mounting the paraffin sections onto slides and the selection of antibody panels, the latter being considered one of the most important steps in the IHC process [11]. Primary and secondary antibodies are used, the primary being either mono- or polyclonal. As polyclonal antibodies derive from several different B-lymphocyte clones, the resulting antibodies are able to bind multiple epitopes, making them fit for cross-platform protein assays where both native and denatured proteins are used, and polyclonal antibodies therefore provide high detection sensitivity. However, they often show cross-reactivity and are difficult to reproduce when immunizing with the same antigen again. Monoclonal antibodies, on the other hand, derive from a single B-lymphocyte clone and the resulting antibodies bind to a single epitope which makes them more specific. However, they are less useful for analysis of proteins in different states, for example denatured proteins. Secondary antibodies are labelled and bind to the primary antibody for visualization of the antigen-antibody immunoreaction in the microscope [11-13].

An important aspect concerning antibodies is their validation, and guidelines have lately, in a joint effort between a group of academic and pharmaceutical based histopathology researchers, been created for validation of antibody biomarkers for IHC assays, with a stepwise approach for the validation process in order to deliver a uniform system that can benefit the entire IHC community, particularly for the validation process not to be repeated [14]. Evaluation of the immunohistochemical staining is based on subjective assessment by the individual pathologist/researcher and is done in a qualitative manner, which may cause problems with analysis since usually there are no reference standards and the decision of interpretation is based merely on the presence or absence of expression. One needs to be certain that a positive result is the same, independently of who is interpreting it and suggestions have been made to replace the microscope with digitization for a more accurate quantitation [11, 15]. However, IHC is commonly used in routine diagnosis of

disease in benign and malignant tissues and has strength over other more quantitative molecular assays in that the expression patterns are visualized in a morphological context, allowing for simultaneous assessment of tissue morphology and antigen localization. As stated by Brandtzaeg, the results do not only identify the cells and tissues but can also tell us something about the function *in vivo*, thus representing a way of ‘talking to the cell’ [16].

The Human Protein Atlas

Initiated in Sweden in 2003 as an extension of the Human Genome Project, the primary aim of the Human Protein Atlas (HPA) project was to generate validated antibodies towards the majority of all human proteins, and systematically explore the distribution and abundance of the human proteome in normal and cancerous human tissues as well as in cancer cell lines [17, 18]. The HPA portal is available to the public as a tool providing high-resolution images of protein expression in normal and cancerous tissues and cells as well as information about antibody validation [18, 19]. The last version of the HPA (12th) encompasses more than 21 900 antibodies, targeting proteins from 16 600 human genes corresponding to around 82% of the human protein coding genes (<http://www.proteinatlas.org>).

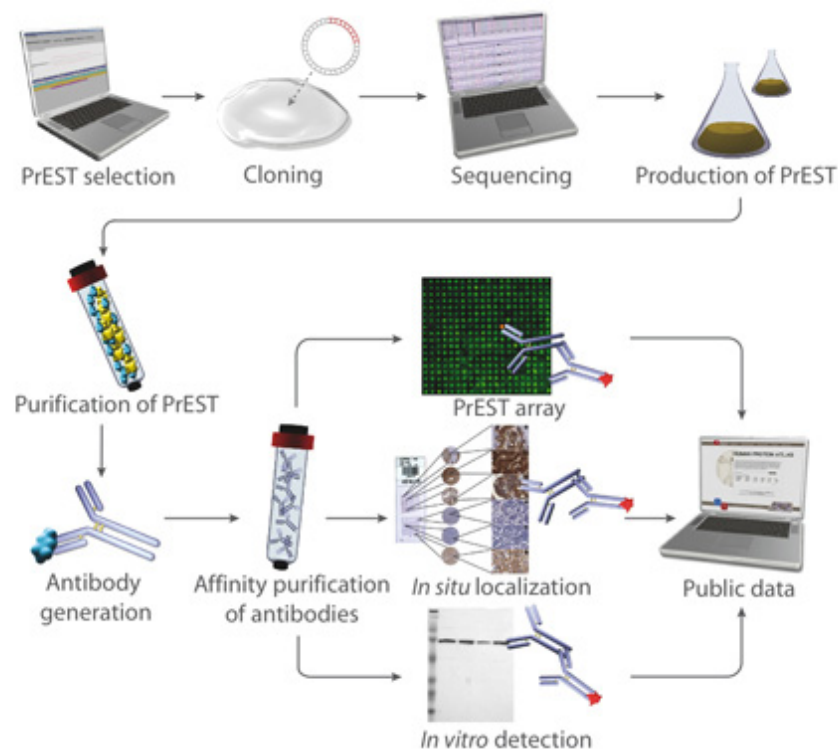


Figure 2. Workflow of the Human protein atlas project. Reprinted with permission from the Human protein atlas project.

One of the challenges of antibody production is to create well-validated antibodies with high selectivity and low cross-reactivity towards other human proteins. Within the HPA project this issue is solved by generation of so called monospecific antibodies, which are produced in a high-throughput manner with strict affinity purification using recombinant protein epitope signature tags (PrESTs) (Figure 2). Each PrEST represents a unique protein region containing 50-150 amino acids with low homology to other human proteins to decrease the risk of cross-reactivity, thus making them suitable for generation of antibodies of high selectivity [20]. Here one takes advantage of the binding specificity of the monoclonal antibodies and the cost-effectiveness of polyclonal antibody production as well as its higher detection sensitivity to create single epitope-specific antibodies. First, an antigen is selected and amplified and the gene fragments are cloned into an expression vector for recombinant PrEST production. The PrESTs are then used as antigens for polyclonal antibody production as well as affinity ligands in the affinity purification process to produce monospecific antibodies [12, 20, 21]. After immunization the antibodies are harvested, affinity purified and thereafter tested on protein arrays and Western blot to ensure the specificity and selectivity of the antibodies. Application specific validation is accomplished by IHC on tissue microarrays (TMA) in order to generate a map of protein expression patterns, and the approved antibodies receive a validation score [19]. The TMAs are constructed from tissues representing the 20 most common types of cancer as well as 48 different normal human tissues. The images and the data are thereafter published on the HPA website.

Tissue microarray technique

As advances are made within the areas of genomics and proteomics, the demand for validation of prospective cancer gene and protein markers has increased. Battifora first described multi-tissue blocks in 1986, and the tissue microarray technique was developed thereafter as a rapid, large scale, high-throughput method for *in situ* discovery of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein targets [22, 23]. The technique is based on a recipient paraffin block into which tumour or normal tissue cores (generally 0.6 - 2 mm in diameter) from a paraffin-embedded donor tissue are inserted (Figure 3). In this manner several hundreds, up to one thousand tissue cores can be inserted into one recipient block and the damage to the donor block from where the tissue is taken is minimized. The TMA block can then be cut in thin sections up to 200-300 times, after which DNA, RNA and protein can be analysed on one microscope glass slide by means of different molecular techniques like IHC, fluorescence *in situ* hybridization (FISH) and mRNA *in situ* hybridization [22, 25].

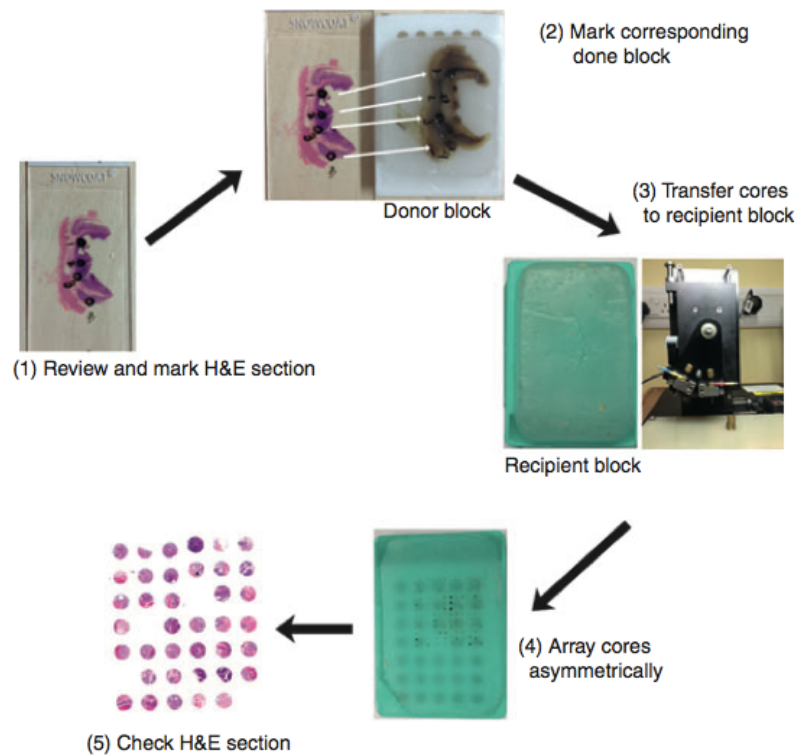


Figure 3. TMA construction. Reprinted with permission from Oxford University Press [24].

One can then, for instance, determine the subcellular localization of the target of interest, the specific cell types expressing the target and eventually connect this to clinicopathological characteristics and survival [25]. Regarding the proper amount of cores, studies have shown that duplicate or triplicate tissue cores are sufficient to accomplish a high degree of concordance to large tissue sections [26, 27]. TMAs have enabled researchers to go from *in vitro* studies of genes, proteins and signalling pathways to the *in vivo* setting. For the extraction of reliable information on the impact of an investigative biomarker on treatment response and survival, annotation of clinicopathological information is important [25]. Since the introduction of the technique, concerns have been raised about TMAs not being representative for the entire tumour e.g. due to tissue heterogeneity. Several studies have however validated the use of TMAs: In one study on breast cancer, Torhorst et al. found that analysis of one single tissue core from each tumour was sufficient to find associations between the molecular markers and clinical outcome and, additionally, that TMAs give equal or even better prognostic information as compared to full-face sections [23]. Similar results were observed in another breast cancer study, where Nocito et al. found that the intra-tumour heterogeneity was averaged out, that tissue from central and peripheral regions of the block gave identical results and that the prognostic associations of the molecular markers were always at least equally good in analysis of the TMAs as compared to whole tissue sections [25]. Another aspect of importance is the reproducibility of results between different laboratories and observers, as different antibodies and staining techniques are used, and the interpretation of the staining may vary. However, in

an inter-laboratory study involving five laboratories, oestrogen receptor (ER) staining was analysed in each laboratory on a breast cancer TMA. It was concluded that TMAs are an effective tool for assessing inter-laboratory variation in ER staining and only one laboratory reported weaker staining for several cores, a finding explained by their using a different antibody and antigen retrieval technique [28]. The TMA technique is more efficient and less expensive than the use of whole tissue sections, since it requires less antibody and reagent. Therefore, it has paved the way for pre-clinical and clinical research within many areas, especially cancer research, where it is a very useful and powerful tool for high-throughput analysis of potential prognostic and treatment predictive biomarkers.

RNA-binding proteins

The central dogma, a term described by Francis Crick in 1958, explains the flow of genetic information as unidirectional, from the nucleotide sequence of the DNA to RNA (transcription) and then into a protein (translation) [29]. This is the simple story but in reality it is a rather complex and tightly regulated process. In summary, in a eukaryotic cell, the specific gene sequence is first transcribed into a primary transcript, pre-messenger RNA (pre-mRNA) (or heterogeneous nuclear RNA, hnRNA), from the DNA by an enzyme called RNA polymerase II and its additional proteins, the transcription factors. The transcription starts at a site called the promoter and ends at the terminator as the DNA and the completed pre-mRNA are released. During the process of transcription the pre-mRNA is modified and undergoes extensive processing in the nucleus of the cell in the form of 5'-end capping and 3'-end poly-A addition as well as splicing (cutting the non-coding introns, leaving only the coding sequences, exons) and only following these steps we have a functional messenger RNA (mRNA). The splicing, performed by a set of RNA molecules, small nuclear RNAs (snRNAs) together forming a spliceosome, allows for a varied collection of mRNAs to be produced from the genome. In the end, only a subset of pre-mRNAs will eventually become functional mRNAs, and a large portion of pre-mRNA will be turned over in the nucleus [29-32]. The mRNAs are thereafter transported to the cytoplasm where the nucleotide sequence is translated into amino acids, together comprising the protein. This occurs on a ribonucleoprotein, ribosome, and the correct amino acids are “carried” there by a transfer RNA, tRNA, which recognizes the specific nucleotides (codon) complementary to the amino acid. The protein is then folded into a three-dimensional structure as it goes through quality control [29].

In the eukaryotic cell, the complex machinery creating proteins from the information encoded in the genes requires extensive regulation. This regulation occurs at several levels, the main being the initiation of RNA transcription [29, 30]. During the past decades, a main focus of research has concerned posttranscriptional gene regulation involving processing of pre-mRNA in the form of splicing, editing and polyadenylation, processes occurring as soon as the pre-mRNAs are transcribed, mediated by various RNA-binding proteins (RBPs) and by small RNAs, e.g. small nuclear ribonucleic particles (snRNPs). These bind to coding or untranslated regions (UTRs) of mRNAs creating ribonucleoprotein

(RNP) complexes and are important determinants of mRNA export, localization, translation and stability [31, 33]. There is a vast amount of RBPs involved in these processes, collectively referred to as heterogeneous nuclear ribonucleoproteins (hnRNPs), all containing one or more RNA-binding domains/motifs (RBD), composed of an amino acid sequence capable of binding to RNA with different RNA-sequence specificity and affinity. The RBPs regulate their target mRNAs in a time and space dependent manner as they associate with the mRNA in different compartments and at different time points [32-35]. The best described RBD is the RNA recognition motif (RRM), also referred to as the RNP motif, consisting of 90 to 100 amino acids with two short sequences, RNP1 and RNP2, as an identifying feature. More than 6000 RRM motifs have already been uncovered and about 1.5-2 % of the human genome is made up of these proteins, which primarily bind to single stranded RNA sequences but have also been found to interact also with other proteins [30, 31, 36]. The RRM motif is present in nearly every organelle of the cell where RNA is found and has unique binding characteristics suggesting multiple functions for the RRM-containing proteins although their main function is participation in pre-mRNA processing [37]. Other well-characterised RNA-binding domains are: K-homology domain, zinc finger and the double stranded RNA-binding domain [30, 31]. Some RBPs have one or more copies of the same or different RBDs creating diversity and assisting in recognizing larger and more complex RNA targets [31, 38]. Through its different RBDs a single RBP may bind to more than one RNA molecule at the same time, for instance a pre-mRNA through one of its RBDs and a snRNA through another [37]. Furthermore, diversity of RBPs is achieved by posttranslational modifications, splicing/alternative splicing and by combining the RNA-binding domain with auxiliary domains and often more than one RBP has the ability to bind to a specific sequence on the target RNA [31]. During different time points in the life of a mRNA various RBPs are bound to it and exert their regulation, and also recruit other regulatory factors and enzymes [35]. New RBPs are continuously being discovered with the help of advances in bioinformatics, biochemical and genetic analyses and, simultaneously, the RNAs to which they bind and interact are being characterised. The RNA-binding domains are used to identify and classify the RBPs, but more knowledge is needed about the interactions between RNA and protein complexes, which is an important step in the further understanding of the function of the RBPs [30, 31].

RNA-binding proteins and cancer

Since RBPs are involved in many aspects of the protein synthesis and regulation of posttranscriptional gene expression they have been an area of interest for research regarding cancer development. As the interaction between the mRNA and the RBPs is highly specific, alterations due to e.g. mutations or overexpression of the RBPs affect the formation of RNPs, in turn causing an impact on the steps of translation involving this specific RBP, for instance aberrant alternative splicing, mislocalization and unregulated translation which might lead to synthesis of pathogenic proteins [39]. For instance, splicing malfunctions are found to be common in development of neurodegenerative disease and cancer, and in the case of cancer, alternative splicing often seems to involve cell-surface expressed proteins. These changes in splicing related to cancer have been proposed to be a potential biomarker for cancer diagnosis and classification [39, 40]. As expression profile changes of RBPs are compared between cancer and healthy states, an increased expression of around 30 RBPs in at least six of the nine cancers studied was found [41]. It is known that many RBPs are aberrantly expressed in cancer cells and that the expression of numerous oncoproteins and tumour suppressor genes are under the control of RBPs [35]. A recent study exploring the expression of RBPs in over 16 different types of human tissues found these to be significantly higher expressed in normal tissue as compared to other proteins and transcription factors, thus stressing their importance in the posttranscriptional regulation [41].

RNA-binding motif protein 3

Background

RBM3 was initially discovered in a human fetal brain tissue complementary DNA (cDNA) library as a novel gene located in the p11.23 region of the human X chromosome during cloning of disease-causing genes. It was found to encode alternatively spliced mRNAs, the longest open reading frame encoding a 157 amino-acid long protein with high sequence similarity to a group of proteins containing an RRM domain, making them able to bind RNA. In addition, RBM3 holds a carboxyl terminal with a high percentage of arginine, glycine and tyrosine [42]. RBM3 belongs to the RNA-binding motif protein family, consisting of ten proteins, all with one to four copies of the RRM domain [37]. Northern blot analysis revealed RBM3 expression in various human adult and fetal tissues, with strong expression in the pancreas, adrenal gland, placenta and testis, whereas no expression was seen in heart or thyroid tissue [42, 43]. Furthermore, when examining the expression of RBM3 in various mouse tissues by Northern blot analysis, a single band of 1.1 kb was detected in all of them [44]. RBM3 binds not only to RNA but also to DNA, and it is postulated that RBM3 might bind to DNA to initiate transcription, followed by binding to nascent RNA to affect its translation [45].

When a cell is facing cold stress, most of the protein synthesis decreases. However, the synthesis of RBM3 increases, even in response to mild hypothermia, and it is one of the first proteins synthesised in response to cold with a maximum increase between 6 and 12 hours [43, 44, 46]. RBM3 is structurally related to another cold inducible RNA-binding protein, the cold-inducible RNA-binding protein (CIRP), both being composed of a carboxyl-terminal glycine-rich domain and one RRM [43]. RBM3 transcripts increase in response to mild cold as tested on various human cell lines by shifting the temperature from 37°C to 32°C, findings which were confirmed in mouse cell lines where RBM3 mRNA levels increased upon exposure to 32°C, in contrast to heat-exposed cells where RBM3 mRNA levels decreased, as seen in cryptorchid mouse testis [43, 44]. The response of RBM3 and CIRP to hypoxia and cold has further been examined in various cell lines, for instance leukemic cells lines, cervical cancer cell lines and human hepatoma cell lines, where both RBM3 and CIRP mRNA transcription as

well as protein expression was found to increased in response to hypoxic conditions, in some cases even with mild hypoxia [47]. RBM3 and CIRP are also stress-inducible proteins participating in the cellular response to oxidative stress [48] and RBM3 transcripts have been shown to increase in human cells in response to protein synthesis inhibitors [43] and decrease in response to serum starvation [49]. RBM3 is further involved in various processes central to cancer biology, e.g. proliferation [49-51], apoptosis [52-54] and angiogenesis [51].

A key step in the control of cold-shock is at the level of mRNA translation, where RBM3 and CIRP are found to bind to various transcripts in the 5'- or 3'-UTR, thus affecting the rate of translation initiation and the stability of the transcript [48]. In addition, alternative splicing of RBM3 affected its localization in neurons and it is suggested that this shift in subcellular localization might indicate a changing role of the protein over time [48, 55]. Overexpression of RBM3 has been proposed to induce global protein translation, presumably by reducing the inhibiting effect of microRNAs on protein synthesis by reducing their levels [56]. RBM3 and other cold-inducible RBPs might also act as RNA chaperones assisting in the processing of RNA molecules in response to cold, thus maintaining translation during cellular stress [55, 57]. However, the fact that most mammalian cells never reaches temperatures of 32°C raises questions about the function of CIRP and RBM3 under normal physiological conditions.

RBM3 and apoptosis

RBM3 has been suggested to have a role in the regulation of apoptosis. This association was seen in a study attempting to identify the pathogenesis behind Huntington's disease, where RBM3 overexpression was found to rescue cells from polyglutamine tract induced apoptosis in both neuronal and non-neuronal cell lines [52], and, in addition, RBM3 was shown to exert hypothermia-induced neuroprotection by decreasing apoptosis in neuronal cells [54]. An association between RBM3 and regulation of apoptosis has also been found in breast cancer [53] and muscle cells, where RBM3 protected cells from apoptosis as well as necrotic cell death [58]. In a study on human embryonal kidney cells, RBM3 overexpression rescued cells from death triggered by serum starvation and as RBM3 was knocked down, cell death was induced, results that would imply that RBM3 is essential for cell survival [49]. In colon cancer cells, knockdown of RBM3 led to apoptosis and induced mitotic catastrophe [51], and in prostate cancer cell lines, silencing of RBM3 and CIRP expression did not induce apoptosis, however, it led to cell cycle arrest in androgen-sensitive but not in androgen-independent cells [59].

RBM3 is expressed in normal as well as in neoplastic cells, but at considerably higher levels in the latter. Expression of RBM3 is also increased in highly proliferating benign cells as compared to resting cells [49]. Immunohistochemical analysis has revealed an increase in RBM3 expression in many malignant tissues, e.g. breast cancer, ovarian cancer, malignant carcinoid, testicular cancer, prostate cancer, colon cancer and non-melanoma skin cancer, and the nuclear staining is often more prominent than the cytoplasmic [49-51, 60].

RBM3 and cancer

Prior to the initiation of this thesis work, the potential value of RBM3 as a prognostic and treatment predictive biomarker had only been described in two human cancer forms, i.e. breast cancer [60] and ovarian cancer [50, 61].

In the breast cancer study, an association between an increased nuclear expression of RBM3 and an improved overall survival (OS) and recurrence free survival (RFS) was found, in particular in ER positive tumours [60]. Similar results were found in a subsequent study on epithelial ovarian cancer, wherein RBM3 was found to be an independent prognostic marker at both the mRNA and protein levels and high RBM3 expression was associated with a significantly improved OS [50]. In addition, RBM3 was found to be a predictor of response to platinum-based chemotherapy *in vitro*, as small interfering RNA (siRNA)-mediated silencing of RBM3 in ovarian cancer cell lines was demonstrated to confer decreased sensitivity to cisplatin [50]. In further studies on epithelial ovarian cancer, an association between RBM3 and a number of cellular processes related to DNA integrity were discovered, and a negative correlation between RBM3 and the DNA damage checkpoint proteins Chk1 and Chk2 was seen *in vitro*. These findings suggest that RBM3 might be involved in the cellular response to DNA damage, and also that RBM3 expression might predict response to platinum-based chemotherapy by silencing of Chk1 and Chk2 [61].

Indirect evidence of an association between RBM3 expression and a favourable prognosis in malignant melanoma had also been provided in a study by Baldi et al., wherein RBM3 was found to be one of five downregulated genes upon progression of melanoma *in vitro* [62].

Seemingly in contrast to the findings of RBM3 expression being associated with an improved survival in human cancer *in vivo*, existing *in vitro* studies suggested an oncogenic role for RBM3. In one study on colon cancer cells, forced expression of RBM3 was demonstrated to confer increased cell proliferation and anchorage-independent growth [51], and in another study on prostate cancer cells mild heat caused a significant downregulation of RBM3 and CIRP, leading to an improved

prostate cancer cell survival and enhanced response to chemotherapeutic treatment in the form of cisplatin or adriamycin [59].

The Hu-antigen R

Background

The Hu-antigen R, HuR, is a ubiquitously expressed protein belonging to the embryonic lethal abnormal vision (ELAV) family of proteins, and its gene is located on chromosome 19p13.2 [35, 63]. HuR encodes a 32kD protein, which regulates gene expression posttranscriptionally through its three RRM, enabling it to bind a large spectrum of target mRNAs with high affinity [30, 35, 64]. In the nucleus it participates in the processing of pre-mRNAs and in the cytoplasm it stabilizes target mRNAs, where it induces, and sometimes even suppresses, their translation [65, 66]. HuR preferentially binds to target mRNAs having U- or AU-rich elements (ARE) in their 5'UTR or 3'UTR, so as to escape their degradation and stabilize them [64, 67]. HuR is known to be associated with cellular processes involving differentiation, response to damaging stimuli and the immune and inflammatory response [64]. Furthermore, as it has the ability to enhance the expression of several anti-apoptotic factors, HuR can be considered a coordinator of a pro-survival program [68]. Findings in another study imply that under persistent stress, when the cell is beyond repair, HuR promotes cell death. Together, these findings suggest that HuR switches from an initial anti-apoptotic function to a pro-apoptotic function in stages where cell death is unavoidable [69].

HuR is predominantly located in the nucleus in unstimulated cells and holds a nucleocytoplasmic shuttling sequence (HNS) in its hinge region, located between RRM2 and RRM3 [70, 71]. The HNS enables it to shuttle between the nucleus and the cytoplasm, as it binds, stabilizes and protects mRNAs from degradation during the transport [67, 70]. An increase in cytoplasmic HuR has been found in malignant cells, both in tissues and in cell lines [72]. Moreover, in response to various stresses, for instance UVB radiation, heat shock and DNA damage, HuR has been found to localize to the cytoplasm [73-75].

HuR and cancer

HuR has not been reported to be mutated in cancer, rather, it seems to affect the expression of target mRNAs by posttranscriptional modifications and is known to be an essential protein involved in cancer-related gene expression [63, 65]. HuR has the ability to regulate the expression of proteins involved in various steps of oncogenesis, e.g. proteins promoting proliferation, inhibiting apoptosis, increasing angiogenesis as well as facilitating invasion and metastasis [64, 65]. Target mRNAs involved in these processes are, to mention a few, cell cycle regulators cyclin A and cyclin B1, cyclooxygenase-2 (COX-2) and the proliferation associated gene c-myc (Figure 4) [76-78].

It has been implicated that HuR and RBM3 have a synergistic ability to increase mRNA stability of key oncogenic proteins, and also that RBM3, similarly to HuR, is a nucleocytoplasmic shuttling protein capable of binding to mRNAs and facilitating their transport, and possibly even loading them onto ribosomes to induce translation [51, 70].

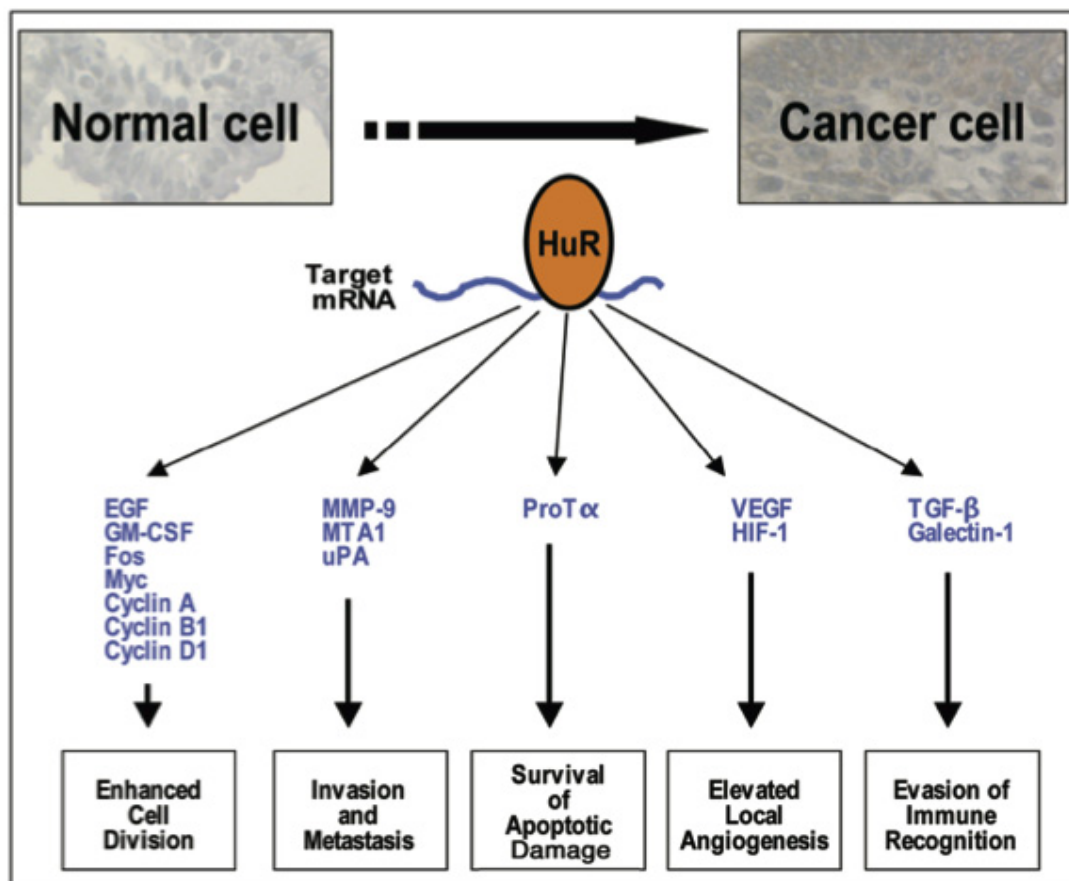


Figure 4. Some of the transcripts that HuR binds to and enhance the expression of. With permission from RNA biology, Isabel López de Silanes et al. [65].

HuR is overexpressed in many types of malignant cells, e.g. in oral cancer cells [79] and lymphoma, and in the latter, HuR was expressed mainly in the nucleus of non-transformed cells but abundantly in the cytoplasm of tumour cells [80]. The same pattern has been observed in immunohistochemical analyses of various other human normal and cancer tissues, e.g. stomach, lung, colon, thyroid and kidney, where HuR was found to be expressed almost exclusively in the nucleus in normal tissue, whereas in cancer tissue, HuR expression was markedly increased in the cytoplasm [81]. Furthermore, overexpression of HuR, in particular its cytoplasmic accumulation, has been correlated with high-grade malignancy and poor clinical outcome in colorectal, ovarian, gastric and breast cancer [82-86]. In a recent study on pancreatic cancer, increased cytoplasmic HuR expression was found to be associated with poor differentiation and it was further revealed that for patients treated with gemcitabine, high cytoplasmic HuR expression was found to correlate with an improved survival [87]. In addition, overexpression of HuR in pancreatic cancer cell lines was found to confer increased sensitivity to gemcitabine. Gemcitabine treatment also significantly increased the cytoplasmic levels of HuR, promoted its association with deoxycytidine kinase (dCK) mRNA, and, moreover, HuR was found to regulate dCK protein levels [87]. As gemcitabine needs to become phosphorylated to become an active metabolite, and the first step of this phosphorylation is executed by dCK, this is the rate-limiting step in the activation of gemcitabine [88, 89]. Consequently, it may be concluded that HuR acts as a key mediator of gemcitabine efficiency [87]. A smaller follow-up study on 24 patients, all receiving gemcitabine, confirmed an improved survival for patients with tumours having high cytoplasmic HuR expression, and proposed that enhancing cytoplasmic HuR expression in tumours with low expression might increase their response to treatment with gemcitabine [90]. However, a recent phase III adjuvant trial with a chemoradiation backbone, encompassing 165 patients with resected pancreatic ductal adenocarcinoma, failed to demonstrate any prognostic or treatment predictive value of cytoplasmic HuR expression [91].

Ki-67

Background

Gerdes et al. initially described Ki-67 in 1983 as they produced a mouse monoclonal antibody that was found to recognize a nuclear antigen present in proliferating cells but missing in resting cells. Moreover, when resting cells were transformed into proliferating cells, expression of Ki-67 was induced, and the opposite was seen when proliferating cells were transformed into resting cells. The name derives from the city of origin, Kiel, and the number of the original clone in the 96-well plate [92]. Ki-67 was further shown to be expressed in the nuclei at all the active phases of the cell cycle (G1, S, G2 and M-phases) but not in resting cells (G0 phase) [93]. In immunoblots of proteins from proliferating cells, the monoclonal Ki-67 antibody detects a double band with molecular weights of 395 and 345 kD, respectively. The antigen to which Ki-67 binds was later identified as a human nuclear protein whose expression was strictly associated with cell proliferation. Since then, it has become a well-established marker in pathology protocols, to measure the growth fraction of cells in human tumours and to determine the growth fraction of a given cell population (Ki-67 labelling index) [94, 95]. IHC is the preferred method of analysis and, most commonly, Ki-67 is scored based on the percentage of tumour cells expressing the protein [95-97].

Of note, despite the progress made in cell cycle research, the exact functional role of Ki-67 is still unknown [95].

Ki-67 and cancer

In the case of multiple myeloma, Ki-67 expression has been found to be a good marker for aggressive disease and to aid in the distinction between multiple myeloma and monoclonal gammopathy of unknown significance (MGUS). Furthermore, Ki-67 has been found to be a prognostic tool in the treatment of patients with soft-tissue sarcoma [98, 99].

In malignant melanoma there is a well-established association between Ki-67 expression and increased tumour thickness [100-102]. Further on, there is an

association between high proliferation rates, as measured by Ki-67 activity, and poor prognosis [101-103]. High Ki-67 has also been shown to be a stronger prognostic factor in thin malignant melanomas than the mitotic rate, the latter being included in the latest version on the AJCC staging manual for localized melanomas [104, 105].

In prostate cancer, Ki-67 has been found to be a potentially useful prognostic marker and an independent significant prognostic factor for disease specific survival (DSS) [106, 107] and cancer specific survival (CSS) [108]. It has however not yet been implemented in clinical protocols.

A vast amount of studies have been performed on Ki-67 and breast cancer and there is increasing evidence that it is an independent prognostic marker for survival and recurrence [96, 109, 110]. Despite this, it is not included as a routine marker in the guidelines by the American Society of Clinical Oncology (ASCO) [111]. A quite recent meta-analysis of 43 studies revealed an association between Ki-67 and a significantly shorter OS and disease free survival (DFS), however, the authors cannot draw any conclusion whether Ki-67 would add any additional prognostic information to the current guidelines [112]. One problem when comparing different studies is the use of various cut-off points and it has been suggested that Ki-67 level above 10-14% should define a high-risk group regarding prognosis [96]. The St Gallen International Expert Consensus recommend the use of Ki-67 and mitosis as well as multigene assays to determine appropriate primary systemic treatment for early breast cancer, in addition to conventional histopathological parameters [113]. Ki-67 is routinely assessed in many clinics and incorporated into clinical protocols as a prognostic marker, however, its role as a treatment predictive marker is still debatable and remains to be further elucidated [96, 97].

Malignant melanoma

Epidemiology and aetiology

In 2008, there was an estimated 200 000 new cases of malignant melanoma worldwide and approximately 46 000 deaths from the disease [114]. It is the sixth most common cancer in the world and the incidence is increasing, in particular in fair-skinned populations [114]. In Sweden and in the Nordic countries, the incidence has been increasing in the past ten years and is expected to continue to rise with an estimated annual number of 1300 new cases for men and women equally [115-118]. Malignant melanoma is the sixth most common type of cancer in Sweden among men and the fifth most common cancer among women [118]. The survival has increased in the Nordic countries as well as in many other European countries, Australia and the US, mainly due to education programmes leading to better public awareness about the importance of skin examination, which leads to an earlier diagnosis [119, 120]. As a matter of fact, the earlier diagnosis is thought to be one of the reasons for the increasing melanoma incidence, since this increase mainly concerns thin melanomas [114, 121].

The aetiology behind malignant melanoma is thought to be a combination of genetic, environmental and individual host factors, as seen in migrant studies in Australia [114, 122]. Risk factors for developing malignant melanoma include numerous common and atypical nevi, a history of sunburn, and exposure to ultraviolet radiation, also from indoor tanning, family history of melanoma, skin phenotype and actinic damage [123-126]. Previous melanoma is also an important risk factor, and exposure to high levels of sunlight in childhood also plays an important role in the development of melanoma, as does intermittent high sunlight exposure [124, 127, 128].

In women, melanomas are more common on the extremities, a location associated with a better prognosis, and in men, location on the trunk, head and neck is more common. Gender is presented as a more important factor for survival than the anatomical location of the lesion, female gender being more prognostic than male [120, 129]. Other important factors indicating an improved prognosis are thickness of the primary lesion (Breslow depth), ulceration, Clark's level of invasion and age of the patient at diagnosis [129, 130].

Pathogenesis and diagnosis

Melanoma of the skin develops through a consecutive step of events starting with benign nevi, dysplastic nevi, radial-growth phase of invasive melanoma, vertical-growth phase of invasive melanoma and ending with metastatic melanoma [128]. Development of malignant melanoma of the skin involves many mutations, the most well known being the mitogen-activating protein kinase (MAPK) signalling cascade involved in cell growth regulation and survival [131]. Activation of this pathway is caused by somatic mutations of the v-raf murine sarcoma viral oncogene homolog (BRAF) and the neuroblastoma RAS viral (v-ras) oncogene homolog (N-RAS), BRAF being mutated in around 50% of melanomas [128, 132]. Further genetic mutations involved in familial melanoma are for instance inactivation of the cyclin-dependent kinase inhibitor 2 (CDKN2) on chromosome 9p21 and inactivation of the phosphatase and tensin homolog (PTEN) on chromosome 10 [133, 134].

As survival is dependent on the thickness of the lesion, early discovery reduces mortality [135]. Common symptoms of malignant melanoma include increased size of the lesion, change in colour, bleeding, a lump at the site of the lesion, itching and/or breakdown of the skin over the lesion [136]. Most commonly the patients themselves or their partner detect the melanoma, however, when the doctor detects the lesion it is usually thinner [135].

Staging of malignant melanoma is done according to the 7th edition of the AJCC staging manual as based on the TNM classification [104]. The T category in the staging is based on the tumour thickness (Breslow depth), ulceration status and mitotic rate, all considered powerful prognostic parameters for patients with localized (stage I or II) melanomas. Sentinel node biopsy is important in order to determine the presence of micrometastases, to decide which patients might benefit from lymphadenectomy. Patients selected for sentinel node biopsy are those with negative nodes clinically and primary lesions between 1-4 mm in thickness [137]. Sentinel node status is part of the AJCC staging system, used as a base for deciding about further adjuvant treatment. In the case of palpable adenopathy, fine needle aspiration biopsy is recommended, and if positive, an x-ray scan, preferably a positron emission tomography computed tomographic (PET-CT) scan, should be done prior to surgical resection to rule out metastatic disease [138, 139]. Patients with positive micrometastases are classified as having stage III disease. Even the finding of a single cell metastasis in the sentinel lymph node can be associated with reduced OS [140]. The M category is defined by the location of the distant metastasis and serum lactate dehydrogenase levels. For melanomas ≤ 1.0 mm the mitotic rate is, next to tumour thickness, the strongest independent prognostic factor [104, 140].

Diagnosis of malignant melanoma includes a biopsy of the primary lesion in whole, with a margin of 1 to 2 mm and shave biopsies should be avoided [104]. For a histologically proven malignant melanoma the recommended excision margins vary according to the size of the lesion. In a Swedish randomized study with long-time follow-up (5-16 years), patients with localized cutaneous melanomas measuring > 0.8 mm with ≤ 2.0 mm thickness were included and randomized to excision with a margin of at least 5 cm or 2 cm. The results showed no significant difference in survival and it was concluded that a 2 cm margin is safe [141]. This finding has further been confirmed in a French study [142] and lately, another Swedish trial concluded that a margin of 2 cm is sufficient for localized cutaneous melanomas thicker than 2 mm [143].

Treatment and prognosis

After resection of the malignant melanoma, some patients remain at risk for recurrence, mainly patients with stage IIB-C and III according to the latest AJCC. These patients are offered treatment with interferon-alpha, although no trials have shown any improvements in OS [144, 145]. However, it has been demonstrated to significantly improve DFS and RFS, and as disease recurrence in patients with malignant melanoma most often results in mortality, one can consider these end points appropriate [144]. Still, only a portion of patients receiving treatment with interferon-alpha experienced a prolonged DFS and RFS and therefore, treatment predictive biomarkers are needed to determine which group of patients actually benefit from this treatment and not only suffers from adverse side effects [146].

For patients with disseminated disease, treatment options include chemotherapy with dacarbazine or temozolomide, both having similar effects on OS, with the latter leading to a longer progression free survival (PFS) and also has the advantage of oral administration [147]. Two new drugs have been introduced lately, i.e. ipilimumab and vemurafenib [148, 149]. Ipilimumab is a fully human monoclonal antibody binding to and blocking the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) to promote antitumour immunity, which has been shown to improve OS in patients previously treated for metastatic melanoma [149]. The second drug, vemurafenib is a potent inhibitor of mutated BRAF, which has demonstrated to increase OS and PFS in previously untreated patients [148].

For patients with invasive melanomas ≤ 1.0 mm in thickness the 10-year mortality is less than 10% and the risk for regional spread is minor [130]. In Sweden the 5-year relative survival is 86.9 % for men and 92.3% for women [150]. According to the 7th edition of the AJCC staging, the 5- and 10-year survival rates for localized melanomas range from 97% and 93%, respectively, for patients with T1aN0M0

melanomas (Stage IA). In contrast, patients with T4bN0M0 melanoma (Stage IIC) have survival rates of 53% and 39%, respectively [104].

Prostate cancer

Epidemiology and aetiology

Prostate cancer is the most common cancer among men in developed countries and the sixth leading cause of cancer death, accounting for 14% of new cancer cases and 6% of cancer deaths in 2008 [1]. Incidence rates vary around the world, mainly due to the variation in utilization of prostate specific antigen (PSA) testing, with the highest incidence in Europe, Northern America and the developed countries of Oceania. Death rates have decreased in the past years in many developed countries [1]. In Sweden the incidence of prostate cancer has been increasing until just recently but is now declining as is the mortality [118, 150].

The only well-established risk factors for prostate cancer are ethnicity, age and family history. As for ethnicity, the incidence of prostate cancer is much higher among men with African descent, most probably ascribed to genetic vulnerability, and a susceptibility region has been found on chromosome 8q24 in a prostate cancer study on African American men [151-153]. As regards age, it is a rare disease among men younger than 40 years and the incidence increases sharply after the age of 55, peaking around 70-74, and thereafter declining slightly [154]. Regarding family history and prostate cancer, many studies have shown an increased likelihood of developing cancer in cases with an affected first-degree relative. This risk has been shown to be at least twice as high, and it increases with an increasing number of affected relatives and with younger age at diagnosis [155, 156]. A large Swedish study has confirmed these findings, and reports the highest hazard ratio, approximately 23, for men <65 years of age with three affected brothers [157]. Other, less well-established risk factors include intake of dairy products and calcium as well as obesity, which has been associated with an increased risk of aggressive prostate cancer [158, 159].

Pathogenesis and diagnosis

As with most cancers, environmental factors play a role also in the development of prostate cancer. However, the main driver of prostate cancer development is the occurrence of *de novo* genetic changes in the prostate itself during the lifetime of a

man (Figure 5) [160]. Several genetic and epigenetic changes playing an important role in the initiation of prostate cancer have been identified, e.g. decreased expression of Glutathione-S-Transferase P1 (GSTP1), an enzyme that decreases oxidative damage in cells [160, 161]. Hereditary prostate cancer, accounting for a quite small portion of the cases, is characterised by early onset of disease (<60 years of age) and three or more first-degree relatives with early onset of prostate cancer [160]. Among the genes wherein mutations have been demonstrated to predispose to hereditary prostate cancer, the tumour suppressor gene BRCA2 is one of the most well known, with mutation leading to a fivefold increased risk of prostate cancer. These patients also tend to have more aggressive cancers with poorer prognosis and are usually offered surveillance [162].

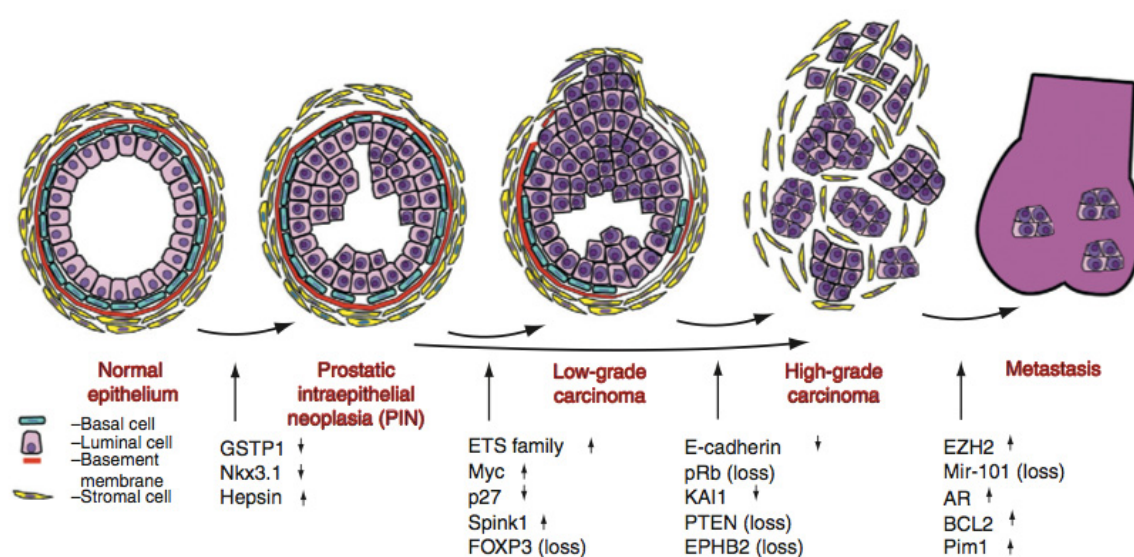


Figure 5. Model of prostate cancer progression. Histological changes and concomitant genetic and epigenetic events during prostate cancer initiation and progression. The deletion or inactivating mutation in tumor-suppressor genes are denoted as (loss). Overexpression of a gene is shown with an arrow pointing up, downregulation of expression is shown with an arrow pointing down. Only initial changes in the expression levels are shown. With permission from Elsevier, Knudsen et al. [160].

During prostate cancer development, changes occur in both epithelial and stromal cells, and prostatic intraepithelial neoplasia (PIN) represents the first morphological step in cancer progression, where high-grade PIN is synonymous to *in situ* carcinoma. Hereafter, many changes occur in the epithelial cells leading to development of adenocarcinoma most commonly in the peripheral zone of the gland [160]. Invasive prostate cancer is divided into high and low grade depending on its architectural growth pattern, that is, the glandular pattern of differentiation. There are five grades, from well differentiated (1) to no glandular structure (5) and the most prevalent pattern and the highest of the remaining patterns are summed up as the Gleason score, whereby a high score signifies more aggressive tumours [153, 160, 163].

Androgens play a central role in the development of prostate cancer and the cancer cells are dependent on androgens in order to survive. Despite lacking evidence of androgens actually initiating carcinogenesis, they are potent mediators of cancer growth and progression, and thus important treatment targets [153, 164].

PSA is an androgen-regulated serine protease that is produced by normal epithelial cells of the prostate as well as prostate cancer cells. It is used as a serum biomarker to detect prostate cancer and also to evaluate treatment response [165]. PSA is tissue-specific but not cancer-specific and since its introduction, its utility as a tool for screening has been under much debate, as not all early detected cancers will become clinically evident, thus leading to over-diagnosis and overtreatment [153, 166]. Recent reviews have not concluded that screening for prostate cancer with PSA would have an impact on overall mortality or death, and there is no evidence to support the use of PSA for routine screening [167]. One way to increase the specificity and sensitivity of PSA is to use age-specific reference ranges with higher references for older than younger men [168]. However, in clinical practice, when men seek a doctor for symptoms related to the prostate or just because they are worried, a PSA-test is performed, followed by a digital rectal examination. If PSA levels are increased to a level where prostate cancer might be suspected, biopsies are taken with help of transurethral ultrasound and staging is done based on the results from the pathological examination of the tissue. According to the TNM classification, five factors are taken into consideration when staging prostate cancer; the growth extent of the primary tumour, spread to lymph nodes, distant metastasis, PSA level and Gleason score [169].

Treatment and prognosis

The treatment of prostate cancer varies according to the stage and risk, and there are many treatment options that need to be discussed between the doctor and the patient. For patients with localized disease, the most common alternatives are radical prostatectomy, hormonal therapy, cryoablation and radiotherapy including brachytherapy. In the case of disseminated disease, treatment options include androgen deprivation, chemotherapy and radiotherapy. Active surveillance, i.e. following the patient until treatment is indicated, or watchful waiting, are also good options for patients with low-grade cancers, low serum PSA, co-morbidity and for older men [153, 170].

Radical prostatectomy is an efficient treatment for patients with localized disease, and one of the most common treatment options. Whether it is superior to watchful waiting or active surveillance is a debated issue, as one Swedish study concluded that patients having undergone radical prostatectomy had an increased survival and lower risk of metastasis as compared to watchful waiting [171], whereas

another study claimed that observation, or sometimes active surveillance, of patients with low PSA and low-risk disease is a better option [172]. Complications from radical prostatectomy include urinary incontinence and impotence [173]. After treatment with radical prostatectomy, 25-40 % will face elevation of serum PSA, a so called biochemical recurrence (BCR), which is defined as two consecutive serum PSA levels ≥ 0.2 ng/mL [173, 174]. In the case of local recurrence, prompt radiation therapy is the treatment of choice [175].

Radiation therapy is also a good treatment option, especially for patients who are considered poor candidates for radical prostatectomy [176]. The various radiation therapies used today are very advanced and allow for minimal damage to the surrounding tissue despite high tumoricidal doses of radiation. Radiation therapy can be combined with hormonal therapy as a curative treatment for locally advanced disease [173].

Hormonal therapy is based on the fact that the prostate cancer is androgen-dependent. Surgical castration or medical castration with oestrogens was found to reduce the size of the cancer and lead to clinical remission in 80% of patients with metastatic disease [177]. Since then, many different hormonal therapies have been developed and are today mainstay in the treatment of metastatic prostate cancer, and also an option for patients with non-metastatic disease [173]. Some of the treatment options available today are; orchiectomy, oestrogens, luteinizing hormone-releasing hormone (LHRH) agonist or antagonist therapy, anti-androgen therapy and androgen deprivation therapy [173].

The relative 5-year survival rate, including all stages of prostate cancer, is almost 100%, the 10-year survival rate is 99% and the 15-year survival rate is 94% [169]. In Sweden the 5-year relative survival is 91.6% and the 10-year relative survival is 82.6% [150].

Upper gastrointestinal cancer

Epidemiology

Oesophageal cancer is the eight most common form of cancer and the sixth leading cause of cancer-related death worldwide. It is most common in developing countries and more common in men than in women [2]. There are two main histological subgroups of oesophageal cancer, squamous cell carcinoma and adenocarcinoma, and although the incidence of adenocarcinoma has increased rapidly in the past years in many Western countries, squamous cell carcinoma still remains the main histological subtype [178-181]. In Sweden, the incidence of both types of oesophageal cancer has slowly increased in the past 30 years, mainly among men, and it represents about 1% of all cancers among men and 0.5% of all cancers among women [150, 182].

Stomach cancer is the fifth most common malignancy in the world, with more than 70% of the cases found in the developing world and is almost twice as common in men as compared to women. Incidence rates vary around the world, mainly explained by differences in diet and prevalence of *Helicobacter pylori* infection, and is highest in Eastern Asia, Eastern Europe and South America. It is the third leading cause of cancer-related death in both sexes worldwide [1, 2, 183]. In Sweden, the incidence of stomach cancer has decreased considerably in the past decades and now barely represents 2% of all cancers [150, 184].

Pathogenesis and aetiology

Chronic irritation and inflammation are found to increase the incidence of squamous cell carcinoma (SCC) and the main risk factors are smoking and alcohol abuse. Many of the patients also have mutations in the TP53 gene [178, 183, 185]. SCC is associated with lower socioeconomic status, and intake of extremely hot beverages has also been found to increase the risk of SCC [178, 186]. Fresh fruits and vegetables as well as aspirin have proven to have a protective effect against developing SCC [178, 183]. For SCC, the main events leading to invasive cancer include conversion of normal squamous epithelium to basal cell hyperplasia, then

to intraepithelial neoplasia (dysplasia and carcinoma in situ) and finally SCC arises [183, 185].

Adenocarcinoma develops mainly in the lower third of the oesophagus and the most important etiological factor is gastro-oesophageal reflux disease (GERD), which is a risk factor for Barrett's oesophagus. In a Swedish study, people with recurring reflux symptoms were found to have an odds ratio (OR) of 7.7 for developing adenocarcinoma, and ORs between 2.5 and 40 have been suggested in various other studies [178, 185-187]. Smoking and overweight also predispose to adenocarcinoma, the latter in part by increasing the risk of GERD. TP53 gene mutations are often present [153, 178, 183]. Fresh fruits and vegetables as well as aspirin intake have been demonstrated to have a protective effect also against adenocarcinoma development [178, 183]. Moreover, *Helicobacter pylori* infection has been suggested to have a protective effect, although results from different studies have shown somewhat contradicting results [188, 189].

Chronic GERD is the main cause of Barrett's oesophagus, characterised by intestinal metaplasia within the oesophageal squamous mucosa. It increases the risk of developing adenocarcinoma, yet, it should be pointed out that only 0.2-1% of individuals with Barrett's oesophagus develop epithelial dysplasia, a preinvasive lesion, and most people with Barrett's oesophagus never develop cancer. The greatest risk has been observed in individuals with longstanding and frequent symptoms of reflux [153, 185, 190].

As for gastric cancer, adenocarcinoma is the main histological type, accounting for over 90% of all gastric cancers [185]. A particular subgroup of tumours originates in the oesophagogastric junction (EGJ), and has their centre within 5 cm proximal and distal of the anatomical cardia. According to the Siewert classification there are three types of EGJ tumours; Type I tumours arise in an area of Barrett's in the distal oesophagus and infiltrates the EGJ from above, Type II tumours arise from cardiac epithelium at the EGJ and Type III tumours arise in the subcardial area and infiltrate the EGJ from below [183, 185, 191]. This classification provides a useful tool for selecting the surgical approach [192]. The aetiology behind gastric cancer is multifactorial but it frequently develops after a long period of atrophic gastritis [185]. The main risk factor is infection with *Helicobacter pylori*, which induces phenotypic changes in the gastric mucosa, initially leading to chronic gastritis and from there to mucosal atrophy, intestinal metaplasia and dysplasia, in turn leading to development of adenocarcinoma. However, since its discovery in the 90's, treatment with antibiotics to eradicate *Helicobacter pylori* has led to a considerably reduced incidence of gastric cancer [183, 185]. Other factors that underlie decreasing incidence of stomach cancer in most parts of the world are the use of refrigerators, decreased intake of salt as well as increased intake of fresh fruits and vegetables [1, 183, 185]. Further risk factors for gastric cancer include adenomatous polyps, chronic gastritis and previous gastric surgery [193].

Diagnosis, treatment and prognosis

The presenting symptoms for many patients with oesophageal cancer are dysphagia and weight loss, as well as pain when swallowing foods or liquids. At this stage the tumour is often locally advanced and may already have spread [186, 193, 194]. The physical examination is usually normal unless the patient has disseminated disease, in which case lymphadenopathy, hepatomegaly and pleural effusions might occur. For further diagnosis, barium swallow study and endoscopy with biopsy are used. Moreover, for patients with disease confined to the oesophagus, examination with an endoscopic ultrasonography might help in determining the depth of invasion, i.e. the T-stage. For assessment of metastatic disease, CT scan and PET are used [186, 193]. For staging, the TNM classification is used, taking into consideration tumour invasion (T-stage) as well as assessment of spread to regional lymph nodes (N-stage) and distant metastasis (M-stage) [195].

More than 50% of the patients have unresectable or metastatic disease at the time of diagnosis [186]. Surgical resection of the oesophagus is the only treatment that can be curative and is offered to patients with localized disease. There are different methods to choose between, depending on the tumour location, the extent of lymphadenectomy required and if neoadjuvant therapy is used. Neoadjuvant chemotherapy is commonly used for patients with T3 or N1 disease, primarily to downstage the disease prior to surgery. Postoperative chemo- or radiotherapy can be offered to patients when tumour cells extend to the surgical margin and when lymph nodes are positive for disease. For patients with unresectable tumours, chemotherapy is used alone or in combination with radiotherapy and, furthermore, most patients need help with pain relief and nutrition and some patients might also need relief from dysphagia with the help of a stent or dilatation [186, 193, 196].

The prognosis for both types of oesophageal cancers has improved, but the 5-year OS is only about 10% [197]. The most important prognostic factors for adenocarcinoma of the oesophagus are depth of mural invasion and presence or absence of lymph node or distant metastasis [185]. For advanced stages of adenocarcinoma, the 5-year OS is less than 25%, in contrast to cases where the tumour is limited to the submucosa or the mucosa, in which case the 5-year survival is up to 80%. For patients with disseminated SCC, the 5-year survival is only 9%, whereas it is 75% for patients with superficial carcinomas [153].

The symptoms of gastric cancer are quite diffuse and vague, initially often mimicking the symptoms of gastritis, which causes a delay in diagnosis with more advanced stages of the tumour. Epigastric pain is the most frequent complaint and some other less common complaints include dysphagia, weight loss and melena [153, 193]. Physical examination most often does not reveal anything unless the disease is advanced when one might palpate an epigastric mass, enlarged liver and

ascites. Diagnosis is made with endoscopy with biopsies for histological evaluation and CT scan is used to determine the presence or absence of distant metastasis [193]. Staging of gastric cancer is based on the TNM classification and depends on the depth of penetration of the primary tumour (T-stage), assessment of spread to regional lymph nodes (N-stage) and distant metastasis (M-stage) [194].

Treatment of gastric cancer in cases with curative intent includes radical surgery with different modalities, and lymph node dissection. However, most patients are not cured by this treatment alone, and the 10-year survival rate for patients with stage 1A disease is only 65% [193, 198]. Pre-, peri- or postoperative chemotherapy is commonly offered, but treatment protocols vary somewhat between different countries. An American study demonstrated a positive effect of postoperative chemoradiotherapy, as compared to surgery alone, and this is offered in many hospitals in the USA. Another European study showed that perioperative treatment with epirubicin, cisplatin and infused fluorouracil, as compared to surgery alone, significantly improved both progression-free and OS [198, 199]. For palliative treatment, chemotherapy improves both quality and quantity of life when compared to best supportive care, and commonly includes a platinum-based compound, e.g. oxaliplatin, in combination with one or more other compounds [200, 201]. Also, for patients with advanced gastric cancer overexpressing the human epidermal growth factor receptor 2 (HER-2), treatment with trastuzumab, a monoclonal antibody, has been demonstrated to lead to a prolonged survival [202].

For gastric cancer diagnosed and treated in an early stage, the 10-year survival after surgery is about 90%. Patients with advanced tumours, with lymphatic and vascular invasion, or more than 15 affected lymph nodes, have a 5-year survival of only 11% [185].

Pancreatic and periampullary cancers

Epidemiology and aetiology

The incidence of pancreatic cancer is increasing and it is now the fourth leading cause of cancer death in the United States [181]. Worldwide, it is the seventh most common cause of cancer related death among men and women [2]. In Sweden, approximately 1000 people were diagnosed with pancreatic cancer in 2011 and it constitutes about 2% of all cancers [150, 203]. Due to the very low 5-year survival rate, the mortality and incidence are almost identical. The incidence of periampullary tumours is lower than for pancreatic cancers and there is a somewhat higher incidence of these tumours among patients with familial adenomatous polyposis [204]. There seems to be a quite long latency between the onset of pancreatic cancer development and diagnosis, a window that could enable earlier diagnosis through screening [205].

The most important risk factor for developing pancreatic and periampullary cancer is smoking, with an at least twofold relative risk, that increases with the duration of smoking and the number of cigarettes smoked daily. Other risk factors include age, chronic pancreatitis, adult onset diabetes and hereditary pancreatitis, as well as dietary factors (high fat and protein, low fruit and vegetable intake), coffee consumption and chemical exposure [183, 185, 204].

Pathogenesis and diagnosis

Ductal adenocarcinoma is the most common cancer of the pancreas and approximately 60% of the cancers arise in the head of the gland, 15% in the body and 5% in the tail, with the remaining 20% occurring diffusely in the pancreas [153, 185]. The development of pancreatic cancer occurs in several steps of genetic changes involving the pancreatic epithelium as a consequence of inherited and acquired mutations in cancer-associated genes. For instance, the tumour suppressor genes TP53 and p16 are inactivated and the oncogene V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) is activated [153, 185]. There are three categories of precursor lesions that may transform into pancreatic adenocarcinoma: pancreatic intraepithelial neoplasia (PanIN), intraductal

papillary-mucinous neoplasm (IPMN) and mucinous cystic neoplasm (MCN). The most common precursor is the PanIN, which can be seen in the ducts of the pancreas. PanIN is further classified into three grades whereby PanIN 1 and 2 are considered as low-grade lesions and PanIN 3 as a high-grade lesion. PanIN is often found next to infiltrating adenocarcinomas, with which they also share a number of genetic alterations. However, only a minority of these progresses to invasive cancer, and high-grade PanIN 3 is most frequently associated with adenocarcinoma. Low-grade PanIN can be found in about 40% of adult pancreas without evidence of invasive carcinoma [153, 185, 206].

Periampullary carcinomas are adenocarcinomas located in or adjacent to the ampulla of Vater, encompassing tumours originating in the pancreas, the ampulla of Vater, the distal bile duct or the duodenum [207, 208]. Pancreatic cancer is the most common periampullary adenocarcinoma, and holds the worst prognosis, while the best prognosis is seen for tumours of duodenal origin [209]. These tumours can be further subclassified as having intestinal or pancreatobiliary type of differentiation, the latter having the poorest prognosis [207, 210].

Adenocarcinoma of the pancreas usually remains silent until its growth invades other structures in its vicinity, and symptoms therefore generally occur rather late and often reflect advanced disease. Pain is often one of the first symptoms, however, by the time the pain appears, the cancer has normally spread beyond cure. Other common symptoms are weight loss and jaundice due to biliary obstruction. Tumours located in the head of the pancreas have a better prognosis as jaundice and pruritus typically occur earlier than when the tumour is located in the body or tail of the pancreas, hence leading to an earlier diagnosis [153, 185, 204]. Upon diagnosis, the pancreatic cancer is often locally advanced with overgrowth onto major vessels close to the head of the pancreas. Due to the delay in diagnosis many patients already have disseminated disease, commonly to the liver, upon diagnosis. Patients with adult onset of diabetes without family history and those with unexplained acute pancreatitis should be evaluated for pancreatic cancer [204].

Diagnosis is initially made with ultrasonography, which also enables diagnosis of dilated bile ducts. However, more precise and useful imaging techniques, also for staging, are provided by CT or magnetic resonance imaging (MRI), both able to visualize the primary tumour as well as possible spread to other organs, e.g. the liver. Endoscopic ultrasonography (EUS) has become a valuable instrument for diagnosing and staging small tumours, and also for obtaining material for histological diagnosis through biopsy [204, 211]. For diagnosis and biopsy of ampullary tumours, endoscopic retrograde cholangiopancreatogram (ERCP) is suitable [204], and for detection of metastatic disease, PET/CT is very sensitive [212].

For patients with symptoms, cancer antigen 19-9 (CA 19-9) is a biomarker that can be used to differentiate between benign and malignant conditions of the pancreas [213]. Nevertheless, it should be used with caution for diagnostic purposes, since conditions like obstructive jaundice for other reasons than cancer may also lead to increased levels of CA 19-9. Moreover, it is not particularly sensitive for detection of early or small-diameter tumours [214]. For patients receiving chemotherapy, it is an important instrument to monitor for early recurrence, if the tumour initially expressed CA 19-9 [214, 215].

Staging is done according to the TNM classification and is performed with the help of diagnostic imaging of the pancreas, most commonly a CT-scan [212].

Treatment and prognosis

Surgery is the only curative treatment for pancreatic adenocarcinoma. At the time of diagnosis, only about 15% to 20% of the patients present with a potentially resectable tumour, and even after complete removal, 5-year survival is only 12% to 20% [204]. The two most frequent types of surgery for cancer in the head of the pancreas are the classic Whipple procedure (pancreaticoduodenectomy) or a pylorus-preserving Whipple operation, both having similar mortality, morbidity and survival rates [216].

Neoadjuvant chemotherapy has not been shown to result in significantly improved survival rates and is usually not recommended [204]. Adjuvant chemotherapy with gemcitabine or 5-fluorouracil (5-FU) plus folinic acid for 6 months has been demonstrated to provide a significant survival benefit as compared with observation for both pancreatic adenocarcinoma and other periampullary tumours, and is now standard treatment [217-219]. On the other hand, adjuvant chemoradiotherapy has failed to show any effect on survival and is generally not recommended [218].

For patients with non-resectable tumours, palliative treatment is offered and most patients will require a stent to relieve biliary obstruction. This can be done either by an endoscopic or by a transhepatic route, or with a surgical biliary bypass [204, 220]. In the case of advanced pancreatic cancer, gemcitabine-based palliative chemotherapy is usually the first line of treatment, however, erlotinib and capecitabine are equally safe and effective options [221]. For patients with good performance status, triple treatment with fluorouracil, irinotecan and oxaliplatin, FOLFIRINOX, is also an option [222].

As mentioned previously, the 5-year survival rate is very poor and the incidence and mortality rates are almost the same. In Sweden the relative 5-year survival is 5.1% for men and 6.0% for women [150, 204].

The present investigation

Aims & background

Prior to this thesis work, the prognostic role of RBM3 in human tumours *in vivo* had only been investigated in ovarian and breast cancer, whereby high RBM3 expression, in particular its nuclear location, was found to be significantly associated with an improved prognosis. In addition, RBM3 was found to confer cisplatin sensitivity in ovarian cancer cells [50, 60].

In this thesis work, we set out to further investigate the longitudinal expression of RBM3 and its prognostic and potentially response predictive role in malignant melanoma, prostate cancer, upper gastrointestinal cancer and pancreatic and periampullary cancer. Moreover, in upper gastrointestinal cancer, the correlation of RBM3 with Ki-67 expression was investigated and in pancreatic and periampullary cancer, we also examined the expression of HuR and its prognostic and response predictive role, as well as the interrelationship between expression of RBM3 and HuR.

Patient cohorts

Paper I

The Malmö Diet and Cancer Study (MDCS) is a prospective population-based cohort study encompassing 28 098 individuals, 11 063 men and 17 035 women, between the ages of 44-74, enrolled between 1991-1996. The main aim of the study was to investigate whether a Western diet rich in fat and low in fruits and vegetables would increase the risk of certain cancer forms. All participants completed a baseline examination including a questionnaire, dietary assessment and anthropometric measures, as well as collection of blood samples. For follow-up, regular matching is being performed with the Swedish Cancer registry regarding cancer incidence and with the National Board of Health and Welfare on data concerning death and cause of death [223, 224]. Until the end of follow-up by

31 December 2008, 264 incident cases of invasive malignant melanoma had been registered in the study population, 226 of which were included Paper I [225].

Paper II

The study comprised an original cohort of 122 patients, from 48-74 years of age, treated with radical prostatectomy for localized prostate cancer at Skåne University Hospital, Malmö, Sweden, between 1998-2003. Histopathological, clinical and follow-up data were obtained from the clinical- and pathology records. Information on vital status and cause of death was obtained from the Swedish Cause of Death Registry up until December 2006 [226].

Paper III

The study comprised a consecutive cohort of 175 patients with oesophageal and gastric adenocarcinomas, all surgically treated at Skåne University Hospital, Malmö and Lund, Sweden, from January 1st 2006 until December 31st 2010. From the original cohort of 303 cases, 128 patients were excluded; all patients who had received neoadjuvant treatment (n=31), cases with metastases from other cancers (n=12), mucosal resections (n=6), consultancies from other departments (n=22), cases with missing archival specimens (n=2) and incorrectly coded cases (n=55). Clinical data, information on recurrence, vital status and cause of death were obtained from the medical charts and the Swedish Cause of Death Registry [227, 228].

Paper IV

The study comprised a retrospective consecutive cohort of 175 patients, surgically treated with pancreaticoduodenectomy for primary pancreatic and periampullary adenocarcinoma at Skåne University Hospital, Malmö and Lund, Sweden, from January 1st 2001 until December 31st 2011. Data on survival were gathered from the Swedish National Civil Register. Follow-up started at the date of surgery and ended at death or at December 31st 2013, whichever came first. Information on neoadjuvant and adjuvant treatment and recurrence was obtained from patient records [229].

Results

Paper I

In this paper, nuclear RBM3 expression and its potentially prognostic value was examined in a cohort of 264 incident cases of primary malignant melanoma in the MDCS. TMAs were constructed from 226 primary tumours and 31 metastases (both regional and distant metastases from various tissues), and, in addition, 25 full-face sections were examined to assess possible heterogeneity.

RBM3 expression could be evaluated in primary tumours from 215 cases and metastases from 31 cases. RBM3 was mainly expressed in the nuclei of the tumour cells, and when present it was expressed in the majority of tumour cells but in varying intensities. Therefore, only the intensity of the staining was taken into consideration in the statistical analyses. RBM3 was also expressed in the cytoplasm in various intensities, primarily in tissue cores with a strong nuclear RBM3 expression, and did therefore not add any prognostic value. The results revealed a clearly higher expression of RBM3 in primary tumours as compared with metastases. There was a significant inverse association between RBM3 expression and depth of invasion, Clark level, clinical stage, mitotic count, nodular vs non-nodular type and ulceration. According to Kaplan Meier analysis, a significantly improved OS ($p < 0.001$) and RFS ($p = 0.020$) were revealed for patients with tumours displaying high RBM3 expression. RBM3 remained an independent predictor of OS but not RFS in multivariable analysis [225].

Paper II

In this paper, nuclear RBM3 expression and its potentially prognostic value was examined in TMAs with primary tumours and paired normal prostate tissue from a cohort of 122 patients with localized prostate cancer.

RBM3 expression in invasive cancer could be evaluated in 88 (72.13%) cases, and was mainly observed in the nuclei of the tumours. Its expression was evidently upregulated in PIN and prostate cancer as compared with benign prostatic glands. There was no significant association between RBM3 expression and conventional clinicopathological parameters. According to Kaplan Meier analysis, a significantly prolonged time to BCR ($p = 0.004$) and PFS ($p = 0.004$) was revealed for patients with tumours displaying high RBM3 expression. RBM3 remained an independent predictor of both BCR and PFS in multivariable analysis. Cytoplasmic RBM3 expression was not prognostic [226].

To evaluate the study for any potential selection bias, basic clinicopathological parameters (Gleason sum, clinical stage, tumour volume, extracapsular extension, seminal vesicle invasion, positive surgical margins and WHO grade) were compared between cases successfully evaluated for RBM3 expression (n=88) and non-evaluated cases (n=34). No significant differences were found between the two groups (data not shown), thus demonstrating absence of selection bias.

Paper III

In this paper, RBM3 expression and its potentially prognostic value was examined in a consecutive cohort of 175 patients with radio- and chemotherapy naive oesophageal and gastric adenocarcinoma. A TMA was constructed with tissue cores from 175 primary tumours as well as matched lymph node metastases from 81 cases, intestinal metaplasia (IM) (gastric IM or Barrett's oesophagus) from 73 cases, normal squamous oesophageal epithelium from 96 cases and normal gastric mucosa from 131 cases. In addition, immunohistochemical expression of Ki-67 was examined.

RBM3 expression could be evaluated in 173 primary tumours and 71 lymph node metastases, as well as in 53 cases of normal squamous oesophageal epithelium, 117 cases of normal gastric mucosa and 72 cases of IM. RBM3 expression was significantly higher in normal-appearing squamous oesophageal epithelium and IM as compared with normal gastric mucosa and primary tumours, and RBM3 expression did not differ between primary tumours and metastases. Furthermore, RBM3 expression was significantly higher in primary tumours ($p<0.001$) and metastases ($p<0.001$) arising in a background of IM. A significant association was found between reduced RBM3 expression and a more advanced T-stage. There was no significant association between RBM3 expression and proliferation, assessed by Ki-67, in primary tumours. However, in metastases, a positive correlation was found between high RBM3 expression and increased Ki-67 expression. Ki-67 expression was not a prognostic factor.

According to Kaplan Meier analysis, a significantly improved OS was revealed for patients with tumours displaying high RBM3 expression in the entire cohort ($p=0.003$) and for patients with radically resected tumours (R0) ($p=0.002$). In addition, RFS was significantly improved for patients with R0 tumours displaying high RBM3 expression ($p<0.001$), and this association was extended to patients with R0 resection and distant metastasis-free disease (M0) ($p<0.001$). RBM3 remained an independent predictor of OS in cases with R0 resection and of RFS in curatively treated patients with R0 resection and M0 disease. Cytoplasmic RBM3 expression was not associated with any clinicopathological parameters and was not found to be prognostic [227].

Paper IV

In this paper, the expression of RBM3 and HuR was examined in TMAs with all primary tumours and 105 paired lymph node metastases from a consecutive cohort of 175 patients with pancreatic and periampullary adenocarcinoma. In addition, a subset (n=50) of matched benign-appearing pancreatic tissues was analysed.

RBM3 expression could be evaluated in 171 primary tumours and 83 lymph node metastases and HuR expression could be evaluated in 172 primary tumours and 88 lymph node metastases. RBM3 expression was mainly evident in the tumour nuclei, whereas HuR was differentially expressed both in the cytoplasm and nuclei. In normal pancreatic tissue, RBM3 and HuR expression could be evaluated in 47 cases, respectively. In the full cohort, RBM3 expression was significantly higher in primary tumours than in normal pancreatic tissue and also higher in the metastases than in the primary tumours. Similar results were seen in the pancreatobiliary but not in the intestinal subgroup of tumours. As for cytoplasmic HuR expression, this was significantly higher in primary tumours than in lymph node metastases, in the entire cohort as well as in the intestinal and pancreatobiliary subgroups, respectively. Nuclear expression of HuR was significantly higher in primary tumours as compared with normal pancreatic tissue, but did not differ significantly between primary tumours and metastases.

Cytoplasmic expression of RBM3 was not associated with any clinicopathological parameters or survival, and therefore all analyses refer to its nuclear expression. A significant positive correlation was found between RBM3 expression and nuclear HuR expression, whereas an inverse correlation was found between RBM3 expression and cytoplasmic HuR expression. In the full cohort, RBM3 expression was not associated with neither OS nor RFS, and similar results were seen for nuclear HuR expression. In the full cohort, there was a trend towards a prolonged RFS for patients with tumours expressing high cytoplasmic HuR and, in patients with intestinal-type tumours, high cytoplasmic HuR was an independent predictor of a prolonged OS.

Further analyses of the potential predictive value of the investigative markers revealed that patients with tumours displaying high RBM3 expression, who had not received adjuvant treatment, had a significantly reduced OS ($p=0.014$) and RFS ($p=0.007$). In contrast, for patients receiving adjuvant treatment, there was a trend towards an improved OS for patients with high RBM3 expression receiving adjuvant treatment ($p=0.070$). When stratifying for treatment with gemcitabine or no adjuvant treatment, patients with tumours displaying low RBM3 expression, not receiving treatment with gemcitabine, had a significantly improved OS ($p=0.020$) and RFS ($p=0.005$), and in contrast, for patients receiving treatment with gemcitabine, high RBM3 expression was associated with a significantly improved OS ($p=0.020$) but not RFS. Furthermore, high RBM3 expression was an independent predictor of improved OS and RFS for patients treated with adjuvant

treatment or gemcitabine. A significant interaction was found between RBM3 expression and adjuvant treatment with regard to both OS ($p_{\text{interaction}}=0.003$) and RFS ($p_{\text{interaction}}=0.009$), and a significant interaction was also found between RBM3 expression and adjuvant treatment with gemcitabine with regard to OS ($p_{\text{interaction}}=0.002$), as well as RFS ($p_{\text{interaction}}=0.002$).

Furthermore, in the pancreatobiliary subgroup, high RBM3 expression was found to be an independent predictor of improved OS and RFS for patients receiving adjuvant treatment or gemcitabine in the.

As for HuR, patients with tumours displaying high cytoplasmic HuR expression, who had not received any adjuvant treatment, had a significantly improved OS ($p=0.010$) and RFS ($p=0.005$). However, in patients receiving adjuvant treatment, no significant associations were found between cytoplasmic HuR expression and survival. When stratifying according to treatment with gemcitabine, patients with tumours displaying high cytoplasmic HuR expression and not receiving treatment with gemcitabine had a significantly improved OS ($p=0.007$) and RFS ($p=0.006$), whereas in patients receiving adjuvant treatment with gemcitabine, no significant associations between cytoplasmic HuR expression and survival were found. A significant interaction was found between cytoplasmic HuR expression and adjuvant treatment with regard to both OS ($p_{\text{interaction}}=0.023$) and RFS ($p_{\text{interaction}}=0.022$), however, no significant interaction was found between cytoplasmic HuR expression and adjuvant treatment with gemcitabine.

Furthermore, in the pancreatobiliary subgroup, high cytoplasmic HuR expression was found to be an independent predictor of reduced OS and RFS for patients receiving adjuvant therapy and, for patients receiving adjuvant gemcitabine, high cytoplasmic HuR was found to be an independent predictor of reduced OS.

Discussion

Translational research related to the role of RBM3 in human cancer is challenging, as current *in vitro* and *in vivo* data appear to be somewhat contrasting, the former suggesting proto-oncogenic features of the protein and the latter strongly indicating its nearly unequivocal role as a biomarker of favourable clinical outcome. At the time when this thesis work was initiated, the prognostic and response predictive role of RBM3 expression in human cancer had only been described in two publications, i.e. related to breast [60] and ovarian [50] cancer.

As regards the scoring system for annotation of RBM3 expression, somewhat different approaches have been used in the different papers. In the first two papers, we used a similar system as in the previously published papers on ovarian and breast cancer, also used for annotation within the HPA project [50, 60, 230].

According to this system, the fraction of nuclear RBM3 expression was denoted as 0 (0-1%), 1 (2-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%), and the intensity of nuclear staining as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). For cytoplasmic staining, only the intensity was accounted for, and denoted as 0 (negative), 1 (weak) and 2 (strong). However, in malignant melanoma (Paper I), in the majority of cases with positive expression, RBM3 was found to be expressed in >75% of the cells. Therefore only the nuclear staining intensity was accounted for. In the last two papers, the nuclear fraction was denoted as the estimated percentage and the nuclear intensity in the same manner as previously. In the last three papers, a nuclear score was calculated by multiplying the fraction and the intensity. The cytoplasmic staining was scored as either 0 (absent) or 1 (present).

In Paper I, RBM3 was dichotomized into high (strong RBM3 expression) and low (negative-moderate RBM3 expression) based on a visual dichotomization of the survival curves for all strata of different nuclear intensities. In Paper II-IV, a combined score of the fraction and intensity was calculated and the optimal cut-off for survival was calculated using classification and regression tree (CRT) analysis (Paper II and III) or the median value (Paper IV). In Paper IV, the cut-off derived from CRT analysis was not prognostic in the entire cohort but yielded similar results regarding the prognostic value of RBM3 expression in treated and untreated patients. In this cohort, the lacking prognostic significance of RBM3 expression in the entire cohort, irrespective of the method used for selection of cut-off, is likely due to its strong predictive value. Therefore, the median value was selected as cut-off, in order to create more equal groups of tumours with high and low RBM3 expression.

An overall issue with scoring of IHC, whether on full-tissue sections or on TMA, is the lack of standardized methods. The fraction can be assessed in various ways, from careful counting of positive cells to an estimated proportion, and the ranges used can be either continuous or subdivisions into percentage ranges. This makes the comparison of results from different research groups and papers quite difficult. The same holds true for the staining intensity. Different systems have been developed in an attempt to overcome scoring discrepancies, e.g. the H-score for ER status and the HercepTest for HER-2 status, and automated approaches have also been suggested to further improve the reproducibility of different systems [231].

The optimal cut-offs for use of RBM3 expression as a prognostic and predictive biomarker remain to be established in forthcoming studies. Since use of a CRT-derived cut off may lead to overfitting of the model, ideally, the same cut-off should be applied in validity studies on independent patient cohorts.

RBM3 expression in primary tumours and metastases

In all four papers, nuclear RBM3 expression has been more prominent than cytoplasmic RBM3 expression, and the latter has not had any significant association with survival or response prediction, which is in line with results from the previous studies on breast and ovarian cancer [50, 60]. In the study on malignant melanoma (Paper I), RBM3 expression was found to be downregulated in lymph node metastases as compared with primary tumours. While this is the first study to describe the expression and prognostic significance of RBM3 in melanoma *in vivo*, the lower expression in metastatic melanoma is in line with a precious *in vitro* study by Baldi et al., in which RBM3 was shown to be one of five downregulated genes upon melanoma progression [62, 225]. In upper gastrointestinal adenocarcinoma (Paper III), RBM3 expression did not differ significantly between primary tumours and lymph node metastases. In contrast, in pancreatic and periampullary adenocarcinoma (Paper IV), RBM3 expression was found to be significantly higher in lymph node metastases as compared with primary tumours. This observation appears to be in line with the finding of RBM3 expression being associated with several unfavourable clinicopathological characteristics and significantly poorer survival in patients not having received adjuvant chemotherapy. Hence, the role of RBM3 in cancer progression may well differ in a tissue and cancer-specific manner, although the vast majority of data seem to confirm its role as a tumour suppressor. Apart from this study, only one other study by Grupp et al., encompassing a large series of prostate cancer patients (n=11152), published in 2014, demonstrated an association between high RBM3 expression and more aggressive tumours, as well as an impaired survival [232]. These findings are not only in contrast with our study on a considerably smaller, but clinically well-characterised, series of prostate cancer patients (Paper II), but also with another paper by Zeng et al., wherein enhanced expression of RBM3 in prostate cancer cells was found to attenuate their stem cell-like features via inhibited splicing of the CD44 isoform CD44v8-v10 [233]. Moreover, in that study, RBM3 expression was found to be downregulated in metastatic as compared with primary prostate cancer at the mRNA level, altogether suggesting an association between decreased RBM3 expression and more aggressive tumours, with an increased metastatic capability [233]. In our study on prostate cancer (Paper II), we compared primary tumours with benign prostate gland epithelium and found that RBM3 was sparsely expressed in the latter but upregulated in PIN and in invasive cancer. However, we did not examine the expression of RBM3 in metastatic prostate cancer. In the study by Grupp et al., RBM3 expression was also found to be upregulated in malignant as compared with benign tissue, but the expression of RBM3 in metastatic tissue was not examined [232].

Another important finding in the study on upper gastrointestinal cancer was the significantly higher RBM3 expression in primary tumours and metastases arising

in a background of intestinal metaplasia as compared to cases without these pre-neoplastic lesions. For tumours in the distal oesophagus and EGJ two different pathways of carcinogenesis, with different immunophenotypic and molecular characteristics, have been suggested: the intestinal pathway, where goblet cells become dysplastic, and the non-intestinal pathway, where the dysplasia arises in cardiac-type glandular mucosa [234, 235]. Moreover, an improved survival has been revealed for patients with adenocarcinoma associated with Barrett's oesophagus as compared to those without this lesion [235]. RBM3 was found to be an independent favourable prognostic factor for patients with upper gastrointestinal cancer (Paper III) and the finding of an association between RBM3 and intestinal metaplasia -associated tumours is therefore of interest, as it suggests that RBM3 might be involved in the intestinal pathway of carcinogenesis.

RBM3 and prognosis

The role of RBM3 as a marker of improved prognosis was confirmed in the first three papers concerning malignant melanoma, prostate cancer and upper gastrointestinal cancer, thereby adding to previous findings in ovarian and breast cancer [50, 60, 61, 225-227]. Meanwhile, another paper has been published, wherein RBM3 expression was demonstrated to be an independent biomarker of favourable prognosis in colorectal cancer [236]. These results were confirmed in two independent patient cohorts (n=270 and n=305, respectively) [236]. These findings appear to stand in contrast to results from a previous *in vitro* study by Sureban et al., wherein RBM3 was suggested to act as a proto-oncogene in colorectal cancer by increasing mRNA stability and translation of transcripts that would otherwise quickly degrade, i.e. COX-2. In addition, forced overexpression of RBM3 was found to significantly increase proliferation and cause colorectal cancer cells to grow in an anchorage-independent manner, whereas knockdown of RBM3 led to mitotic catastrophe [51]. In that study, immunohistochemical expression of RBM3 was also analysed in a few samples of colorectal cancer tissue. While the scoring system was not clearly denoted, the authors claimed to observe an upregulated expression of RBM3 in cancerous as compared with normal tissue, and an increased expression in tumours of more advanced stages. Also at the mRNA level, a stage-dependent increase of RBM3 was observed in malignant as compared with benign tissue. However, the relationship of RBM3 expression with prognosis was not explored [51].

In 2013, in another study from our research group, the prognostic value of RBM3 expression was examined in a cohort of 343 patients with urinary bladder cancer. The results demonstrated that high nuclear RBM3 expression was an independent predictor of a prolonged OS and DSS. For patients with non-muscle invasive, i.e. pTa and pT1 tumours, high RBM3 expression was associated with a significantly prolonged time to disease progression [237].

RBM3 and proliferation

Several studies suggest RBM3 to be associated with proliferation *in vivo* and *in vitro*. For instance, high RBM3 expression has been observed in proliferating zones in the adult rat brain tissue and, in the same study, strong RBM3 staining was found in cells expressing Ki-67 [238]. As mentioned previously, increased proliferation has also been observed in colon cancer cell lines upon forced overexpression of RBM3, whereas downregulation of RBM3 in prostate cancer cell lines significantly inhibited proliferation [51, 59]. Moreover, RBM3 expression has been shown to increase in highly proliferating benign tissue, i.e. intestinal epithelial cells, as compared to resting cells, and knockdown of RBM3 in human embryonal kidney cells led to decreased proliferation [49]. The finding of a significantly higher expression of RBM3 in normal squamous oesophageal epithelium as compared with normal gastric mucosa in Paper III may also reflect the more proliferative nature of this tissue, although Ki-67 expression was only annotated in the invasive and metastatic tissues [227].

The possible association between RBM3 and Ki-67 expression has been further investigated in the malignant melanoma cohort (Paper I), in another study by our research group [239]. While no significant association was found between expression of RBM3 and Ki-67 in primary malignant melanoma, a significant inverse correlation was found between RBM3 expression and another proliferation marker, the minichromosome maintenance 3 (MCM3) protein [239]. An inverse correlation between RBM3 and MCM3 has previously been observed in epithelial ovarian cancer *in vivo* and *in vitro*, and in line with this observation, high tumour-specific MCM3 expression was associated with a poor prognosis for ovarian cancer patients [61]. In our melanoma cohort (Paper I), high Ki-67 expression was found to be associated with reduced OS and DFS in the entire cohort, and to be an independent prognostic factor for reduced DFS and OS in men but not in women [240]. This finding confirms the well-established association between high Ki-67 and poor prognosis for malignant melanoma patients, as mentioned previously [101, 102].

In the study on upper gastrointestinal cancer (Paper III), Ki-67 expression was examined in primary tumours and metastases, and while no association between the biomarkers was found in primary tumours, there was a significant association between higher RBM3 expression and proliferation in metastases. The expression of Ki-67 in upper gastrointestinal tumours, especially in metastases, has not been that widely examined, and the relevance of this finding is therefore not so clear, especially since Ki-67 expression in primary tumours was not significantly associated with clinical outcome [227].

RBM3 and HuR

In pancreatic and periampullary cancer (Paper VI), a significantly positive correlation was found between nuclear RBM3 expression and nuclear HuR expression, whereas a significantly negative correlation was found between nuclear RBM3 expression and cytoplasmic HuR expression. The possible link between these two proteins has previously been investigated in colorectal cancer, and it has been suggested that they might have a synergistic ability to increase mRNA stability of key oncogenic proteins, e.g. COX-2. The proteins were also found to co-localize, predominately in the nucleus, and RBM3, like HuR, was proposed to be a nucleocytoplasmic shuttling protein [51]. Previously, overexpression of HuR, in particular its cytoplasmic accumulation, has been correlated with high-grade malignancy and poor clinical outcome in colorectal, ovarian, gastric and breast cancer [82-86]. On the contrary, high RBM3 expression, in particular its nuclear accumulation has been associated with an improved survival in many cancer forms [50, 60, 225-227, 236, 237]. These results support the finding in our study of an inverse relation between nuclear RBM3 expression and cytoplasmic HuR expression in pancreatic and periampullary cancer.

RBM3 and response prediction

In the last study on pancreatic and periampullary cancer (Paper IV), no significant association was found between RBM3 expression and prognosis in the entire cohort, and RBM3 was associated with less favourable clinicopathological characteristics. This finding stands in contrast to previous studies on RBM3 expression and survival, where RBM3 has been found to be an independent predictor of favourable outcome [50, 60, 225-227, 236, 237]. However, high RBM3 expression was found to be an independent predictor of response to adjuvant chemotherapy, in particular regimens including gemcitabine. This observation is well in line with the previous finding of RBM3 being predictive of response to platinum-based chemotherapy in epithelial ovarian cancer *in vitro*, and presumably also *in vivo*, since the majority of patients with epithelial ovarian cancer are given adjuvant, platinum-based, chemotherapy [50]. The finding of RBM3 being response predictive also held true in subgroup analysis of pancreatobiliary type tumours, but not in intestinal type tumours. This is a very important finding, as it is well established that the prognosis for patients with pancreatobiliary type tumours is worse than for patients with intestinal type tumours, and they are therefore likely to have a greater benefit from more aggressive adjuvant chemotherapy [207]. In addition, since there was a slight overrepresentation of patients with intestinal type tumours in the group with low RBM3 expression, it is of substantial value that these findings remained

significant also in the pancreatobiliary subgroup. Regarding adjuvant treatment for patients with periampullary tumours, a large randomized controlled trial encompassing 287 patients showed a survival benefit for patients who received treatment with 5-FU or gemcitabine in multivariable analysis [219]. However, not all patients benefit from this treatment and, thus, there is an urgent need to find biomarkers for a more accurate prediction of which patients will actually benefit from adjuvant chemotherapy and not only experience adverse side effects.

One advantage of our study is the almost equal proportion of patients who received adjuvant treatment and patients who did not receive any adjuvant treatment, in particular in the pancreatobiliary subgroup (51/110). Therefore, although predictive biomarkers are best evaluated in tumours from randomized trials, this retrospective cohort provides a comparatively strong setting for identification of biomarkers with a response predictive value.

HuR and response prediction

In the last study on pancreatic and periampullary tumours (Paper IV), we also examined the prognostic and predictive value of HuR. While no significant associations were found between HuR expression and prognosis in the full cohort, high cytoplasmic HuR expression was significantly associated with a prolonged OS and RFS in patients who did not receive adjuvant treatment or treatment with gemcitabine. As mentioned previously, in two smaller studies on patients with pancreatic cancer, low cytoplasmic HuR expression was demonstrated to correlate with an impaired survival for patients treated with gemcitabine [87, 90]. However, in both these studies, all patients received treatment with gemcitabine and no comparison was done with patients who did not receive any chemotherapy. Lately though, in a phase III adjuvant trial with a chemoradiation backbone, encompassing 165 patients with pancreatic ductal adenocarcinoma, cytoplasmic HuR was not found to be prognostic or treatment predictive [91]. Further on, it was observed in our study that cytoplasmic HuR expression was significantly higher in intestinal type tumours than in pancreatobiliary type tumours, and in the analysis of the full cohort, two thirds of the patients with intestinal type tumours fell into the category of high cytoplasmic HuR (42/63). Since these patients have a better prognosis than patients with pancreatobiliary type tumours, this might affect the results, as more patients with better prognosis fall into the category of high cytoplasmic HuR expression in the analysis of the full cohort. Therefore, pancreatobiliary type tumours were examined separately, and a significantly impaired OS, but not RFS, was then observed for patients with tumours having high cytoplasmic HuR expression receiving adjuvant therapy and for those receiving gemcitabine, findings which held true also in multivariable analysis.

Further on, the median value for cytoplasmic HuR expression used in the subgroup analyses was the median for cytoplasmic HuR expression in the full cohort. We therefore also applied the median values for the pancreatobiliary and intestinal subgroups, respectively, to see whether this would alter the results. By this approach, in the intestinal subgroup, the significantly improved OS and RFS seen for patients with tumours expressing high cytoplasmic HuR not receiving any adjuvant treatment or treatment with gemcitabine, remained significant only for RFS (data not shown). In the pancreatobiliary subgroup, the RFS for patients with high cytoplasmic HuR receiving adjuvant therapy became significant (HR 1.81, 95% CI 1.01-3.25) (data not shown).

Major strengths and limitations

In this thesis work there are some aspects that merit further attention. As regards the choice of antibody, the same mouse monoclonal antibody against RBM3 has been used in all papers. The specificity of this antibody has been extensively validated using Western blot and IHC in ovarian and colorectal cancer cell lines, including siRNA-mediated knockdown of RBM3 [50, 236]. In the study on RBM3 and breast cancer, a polyclonal antibody was used, which has been further validated in the TMA of the ovarian cancer cohort with concordant results [50, 60].

As regards the use of TMA as a tool for research, matters of tissue heterogeneity and reproducibility have been discussed previously [23, 25, 28]. In all the studies included in this thesis, two to three tissue cores from the primary tumour have been sampled, and when possible, tissues have been sampled from different blocks of the primary tumour and metastases. For normal tissue, one to three cores have been sampled. In our study on malignant melanoma (Paper I) the IHC staining of RBM3 was also analysed on 25 full-face sections, and the results showed an excellent concordance with the TMA-based analysis.

IHC has its advantages alongside other methods in that it is fast and simple, and enables assessment of protein expression in different subcellular compartments. As regards RBM3 expression, it is evident that its nuclear location provides the best prognostic and predictive information. Ideally, the prognostic and/or predictive value of an investigative biomarker should be confirmed both at the protein and mRNA levels, although these do not necessarily correlate. In the study on ovarian cancer, RBM3 expression was demonstrated to be prognostic both at the protein and mRNA levels, although not in tumours from the same patient cohort [50]. We have not been able to find publically available datasets on melanoma, prostate or upper gastrointestinal cancer from which information on the clinicopathological or prognostic correlates for mRNA levels of RBM3 could be

retrieved. The finding of a downregulated expression of RBM3 at the mRNA level in an *in vitro* model of melanoma progression [62] are however in line with the findings from Paper I. In a publically available dataset encompassing 102 cases of pancreatic cancer [241], RBM3 was not found to be prognostic at the mRNA level (unpublished observation). However, as treatment data were not available, no stratified analysis could be performed. Therefore, the lack of prognostic value for RBM3 at the transcriptional level cannot be considered to contradict the findings in our cohort of pancreatic and periampullary cancer, wherein no prognostic value could be demonstrated for RBM3 protein expression in the unstratified analysis.

Conclusions

The results from this thesis work can be summarized as follows:

- High RBM3 expression is an independent factor of improved survival for patients with malignant melanoma, prostate cancer and oesophageal and gastric adenocarcinomas (Paper I-III).
- RBM3 expression is downregulated in metastatic as compared with primary melanoma (Paper I).
- RBM3 expression is upregulated in PIN and invasive prostate cancer as compared with benign prostate gland epithelium (Paper II).
- RBM3 expression is significantly higher in benign squamous oesophageal epithelium than in benign gastric mucosa (Paper III).
- RBM3 expression is significantly higher in Barrett's oesophagus and gastric intestinal metaplasia than in invasive and metastatic upper gastrointestinal cancer (Paper III).
- RBM3 expression does not differ significantly between invasive and metastatic upper gastrointestinal cancer (Paper III).
- In oesophageal and gastric adenocarcinomas, RBM3 expression is significantly higher in primary tumours and metastases arising in a background of IM compared with cases without IM (Paper III).
- RBM3 expression is upregulated in pancreatic and periampullary cancer as compared with normal pancreatic tissue (Paper IV).
- In pancreatic and periampullary cancer, RBM3 expression is significantly upregulated in lymph node metastases as compared with primary tumours (Paper IV).

- In pancreatic and periampullary cancer, cytoplasmic HuR expression is significantly downregulated in lymph node metastases as compared with primary tumours (Paper IV).
- In pancreatic and periampullary cancer, RBM3 expression correlates positively with nuclear HuR expression and inversely with cytoplasmic HuR expression (Paper IV).
- In pancreatic and periampullary cancer, high RBM3 expression is an independent negative prognostic factor for patients not receiving adjuvant chemotherapy, and an independent favourable prognostic factor for patients treated with adjuvant chemotherapy (Paper IV).
- In pancreatic and periampullary cancer, high cytoplasmic HuR expression is significantly associated with a prolonged survival in patients not receiving adjuvant treatment (Paper IV).

Future perspectives

As the incidence of cancer and cancer-related deaths are constantly increasing worldwide, there is an urgent need for new early diagnostic, prognostic and predictive tools. Cancer is however not one but a very heterogeneous group of diseases, even within each given organ and tissue type from which it arises. Therefore, despite major research advances in the wake of the –omics revolution, and the continuous introduction of novel targeted therapies, treatment protocols are far from sufficiently “personalised” for the majority of cancer patients. Moreover, it is becoming increasingly evident that not only the tumour but also individual factors may have considerable impact on the prognosis as well as response to systemic treatment. Molecular Pathological Epidemiology (MPE), first proposed in 2010, is an emerging multidisciplinary research field that investigates the relationship between exposure factors with molecular signatures of the tumours [242]. Such an approach may add some important pieces to the puzzle and help us gain further insight into mechanisms related to cancer initiation and progression, with the ultimate goal to design optimized strategies for personalised prevention and therapy. Of note, the findings in Paper I are based on incident melanomas in the Malmö Diet and Cancer Study. Although we did not continue down the road to further investigate the associations of RBM3 expression with lifestyle-related factors in this thesis, such studies have now been enabled, and may indeed add additional insight into the role of RBM3 in the initiation and progression of melanoma.

The prognostic and treatment predictive value of RBM3 expression needs to be validated in tumours from additional retrospective as well as prospective patient

cohorts, ideally also including randomized treatment trials. Apart from the adjuvant situation, RBM3 may also prove to be a valuable biomarker for identification of patients with disseminated cancer at diagnosis who will respond particularly well to certain chemotherapy regimens, and thus become long-term survivors. An initial approach to test this hypothesis could be to identify long-term survivors with advanced cancer at diagnosis or any long-term survivors diagnosed with pancreatic cancer in the Swedish Cancer Register, and then compare RBM3 expression in tumours from cases and matched controls. Moreover, while it may be difficult to refrain from giving adjuvant chemotherapy to patients with pancreatic cancer, it would be of interest to examine whether RBM3 may be a useful biomarker for identification of patients with borderline resectable tumours who will respond well to neoadjuvant chemotherapy, and thus become resectable.

Although the results from this thesis further consolidate the potential clinical utility of RBM3 as a prognostic and/or predictive biomarker in several types of human cancer, the functional basis for these observations is far from understood. Therefore, it will also be highly relevant to follow up with further *in vitro* studies, not least in order to gain further insight into the mechanisms underlying the observed associations of RBM3 expression with increased sensitivity to various chemotherapies *in vivo*.

Populärvetenskaplig sammanfattning

Allt fler människor drabbas av cancer, som idag är den ledande dödsorsaken globalt. De vanligaste cancerformerna i världen är lungcancer, bröstcancer och tjocktarmscancer och det är störst risk att dö i lungcancer, levercancer och magcancer. Även i Sverige ökar antalet cancerfall, till stor del på grund av att vi lever allt längre och cancer är idag en av våra stora folksjukdomar. Man räknar med att minst var tredje person i Sverige kommer att få en cancerdiagnos under sin livstid. De vanligaste formerna av cancer i Sverige är bröstcancer hos kvinnor och prostatacancer hos män. Andra vanliga cancerformer är hudcancer (exklusive malignt melanom) samt tjocktarmscancer.

Vår kropp är uppbyggd av celler och i cellkärnan finns vårt DNA som kan ses som ett "bibliotek" där vår arvs massa, d.v.s. våra gener, förvaras. Varje gen bär på en kod utifrån vilken proteinet tillverkas när behov uppstår. Tillverkningen av ett protein börjar med att dess gensekvens kopieras från DNA till RNA i cellkärnan. Därefter transporteras RNA-molekylen ut från cellkärnan till cytoplasman, d.v.s. utrymmet mellan cellmembranet och cellkärnan, där den översätts till ett protein i de s.k. ribosomerna, cellens proteinfabrik. Arvs massan är densamma i alla våra celler men proteinerna som tillverkas varierar mellan olika celler och olika organ. Proteiner är involverade i otaliga processer i våra celler och ute i kroppen, bland annat i form av enzymer, transportproteiner och lagringsproteiner. Proteiner utgör även en viktig del av immunförsvaret, inte minst i form av antikroppar. Det finns även proteiner som reglerar vilka gener som ska tillverkas. Cellerna i våra kroppar delar sig konstant och ett av de viktigaste stegen innan celldelningen kan ske är när vår arvs massa dupliceras. Celler dör också på ett noggrant reglerat sätt, bl.a. genom så kallad apoptos. När celler av olika anledningar förlorar sin förmåga till reglerad celldöd och börjar dela sig ohämmat omvandlas de till cancerceller. Detta föregås av flertalet förändringar i arvs massan, så kallade mutationer, som exempelvis kan uppstå vid felaktig duplicering av arvs massan i samband med celldelningen.

Det finns idag flera sätt att diagnostisera och klassificera cancer. Att mäta nivån av ett specifikt protein i blodet kan vara ett sätt, ett annat tillvägagångssätt är att ta ett vävnadsprov från tumören och undersöka detta i mikroskopet. Man får då en uppfattning om vad för slags tumör det rör sig om och hur elakartad den är, men i vissa fall även information om dess förmåga att svara på olika typer av behandling och hur snabbt den riskerar att återkomma efter behandling.

Man vet idag att även om cancerceller liknar varandra i mikroskopet är det ändå en väldigt stor skillnad på deras inneboende aggressivitet, dvs. förmåga att sprida sig, samt känsligheten för olika cellgiftsbehandlingar. Det finns således ett stort behov av förfinad diagnostik för att kunna ge rätt behandling till patienter med mer elakartade tumörer samt undvika överbehandling av patienter med mindre elakartade tumörer. Trots de framsteg som gjorts inom cancerforskningen under de senaste decennierna finns det ännu alltför få kliniskt användbara s.k. ”biomarkörer”, som kan hjälpa till att identifiera viktiga biologiska särdrag hos enskilda tumörer.

RBM3 är ett protein som har förmåga att binda till både DNA och RNA. Det är ett protein som uttrycks i samband med kyla och annan stress som cellerna kan utsättas för. Dess funktion är inte helt kartlagd, men man vet att det är ett protein som har förmågan att binda till och påverka vilka andra proteiner som ska tillverkas i cellen.

Innan detta avhandlingsarbete påbörjades hade uttrycket av RBM3 endast undersökts i bröstcancer samt äggstockscancer. Resultaten från studierna, som utfördes av vår forskargrupp, visade att patienter vars tumörer uttryckte höga nivåer av RBM3 i cellkärnan hade betydligt bättre överlevnad än patienter vars tumörer hade låga nivåer av RBM3. I studien om äggstockscancer fann man även en koppling mellan höga nivåer av RBM3 och bättre svar på cellgiftsbehandling.

Syftet med denna avhandling har varit att närmare undersöka uttrycket av RBM3 och hur detta påverkar prognosen och svar på behandling i några andra av våra vanligaste cancerformer. För detta ändamål har vävnad från tumörerna undersökts i mikroskopet med hjälp av s.k. immunhistokemisk analys, där antikroppsbundet protein visualiseras med hjälp av antikroppar.

I det första delarbetet undersöktes uttrycket av RBM3 i primärtumörer (modertumörer) och metastaser (dottertumörer) från 215 patienter med malignt melanom. Vi fann att nivåerna av RBM3 var högre i primärtumörerna än i metastaserna. Vi fann även att patienter vars primärtumörer hade höga nivåer av RBM3 i cellkärnan levde betydligt längre efter diagnos än de patienter vars tumörer helt saknade eller hade låga nivåer av RBM3.

I det andra delarbetet undersöktes uttrycket av RBM3 i primärtumörer och normalvävnad från 88 patienter med prostatacancer. Vi fann förhöjda nivåer av RBM3 i vävnad med förstadier till prostatacancer och i cancervävnad jämfört med normal prostatavävnad. Vi fann även att patienter vars primärtumörer hade höga nivåer av RBM3 i cellkärnan levde betydligt längre efter diagnos än de vars tumörer helt saknade eller hade låga nivåer av RBM3.

I det tredje delarbetet undersöktes uttrycket av RBM3 i primärtumörer och metastaser, samt normalvävnad och förstadier till cancer, från 173 patienter med cancer i matstruben och magsäcken. Vi fann betydligt förhöjda nivåer av RBM3 i

normal matstrupsvävnad och i vävnad med förstadiieförändringar till cancer jämfört med normal magsäcksvävnad och vävnad från primärtumörer. Patienter vars primärtumörer hade höga nivåer av RBM3 i cellkärnan levde dessutom betydligt längre efter diagnos än de vars tumörer helt saknade eller hade låga nivåer av RBM3.

I det sista delarbetet undersöktes uttrycket av RBM3 i primärtumörer och metastaser, samt normalvävnad, från 171 patienter med bukspottkörtelcancer. Vi fann betydligt förhöjda nivåer av RBM3 i metastaser jämfört med primärtumörer, medan RBM3 inte alls uttrycktes i normal bukspottkörtelvävnad. I denna studie fann vi ingen koppling mellan RBM3 och längre överlevnad efter diagnos när vi gjorde överlevnadsanalyser av hela patientgruppen. Däremot fann vi att patienter som inte fått cellgiftsbehandling och vars tumörer uttryckte höga nivåer av RBM3 levde betydligt kortare än obehandlade patienter vars tumörer hade låga nivåer av RBM3. Omvänt sågs hos de patienter som fått cellgiftsbehandling och vars tumörer hade höga nivåer av RBM3 en betydligt bättre överlevnad jämfört med de behandlade patienter vars tumörer hade låga nivåer av RBM3. I denna studie undersöktes även uttrycket av ett annat RNA-reglerande protein, HuR, i 172 patienter. I motsats till RBM3 var uttrycket av HuR betydligt högre i primärtumörerna än i metastaserna. Liksom RBM3 var uttrycket av HuR inte heller kopplat till överlevnad i hela patientgruppen. Däremot fann vi att patienter som inte fått någon cellgiftsbehandling och vars tumörer hade höga nivåer av HuR levde betydligt längre än patienter vars tumörer hade låga nivåer av HuR.

Sammanfattningsvis har vi kunnat visa att höga nivåer av proteinet RBM3 i malignt melanom, prostatacancer, och cancer i matstrupen och magsäcken är kopplat till förbättrad överlevnad efter diagnos. Detta innebär att RBM3 skulle kunna bli en användbar biomarkör för att identifiera dels patienter med god prognos (høgt tumöruttryck av RBM3), som därmed inte behöver cellgiftsbehandling, och dels patienter med sämre prognos (långt tumöruttryck av RBM3), som behöver mer intensiv behandling för sin cancersjukdom. För patienter med bukspottkörtelcancer har vi däremot funnit att RBM3 är en lovande biomarkör för att identifiera de patienter som har störst nytta av cellgiftsbehandling. Fler studier krävs dock för att bekräfta dessa fynd.

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