Potential Immune Biomarkers in Gastrointestinal Cancers:

Immune Inhibitory Molecules, Lymphocytes and Tumor Antigens

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Cover: $CD8^+$ immune infiltrating cells (brown) surrounding a metastatic colorectal cancer tumor (lower left) to the liver (upper right). Very few immune cells penetrate into the tumor. Circular areas symbolize tissue microarray cores on a glass slide.

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Potentiële immuun biomarkers in gastro-intestinale tumoren: immuun-remmende moleculen, lymfocyten en tumor-antigenen

Thesis

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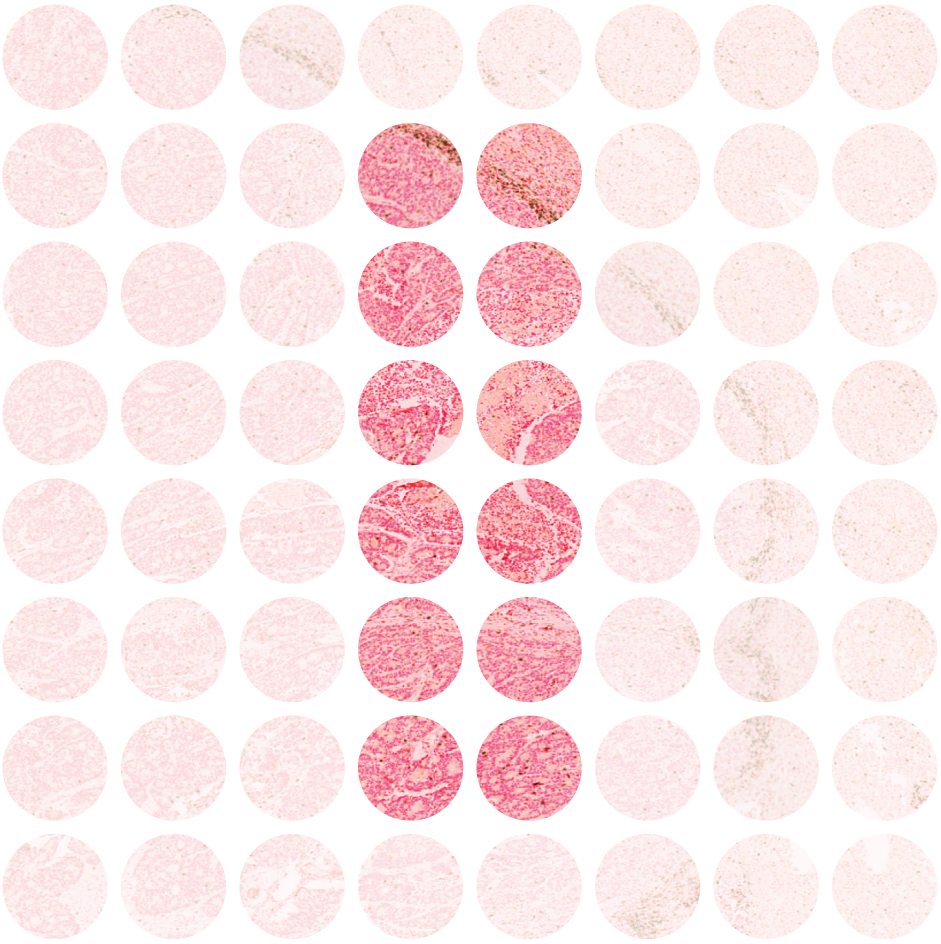
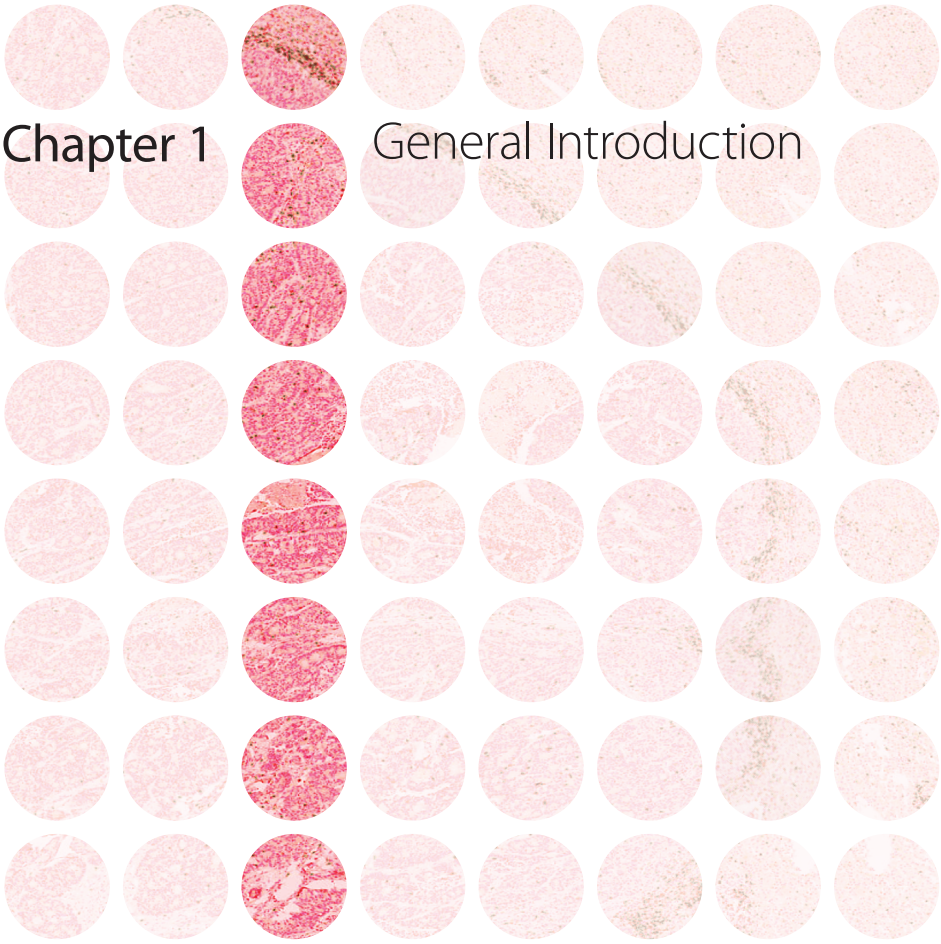


PART I

INTRODUCTION



Chapter 1 General Introduction

GENERAL INTRODUCTION

Tumor immunology: an evolutionary perspective

The elegant laws of natural selection (1) have guided life from single celled organisms to increasingly complex species. The existence of complex multicellular biology requires the preservation of homeostasis, which means individual cells behave in a way that is beneficial to the whole. However, individual cells are not immune from the laws of natural selection (2). Thus, if the constrains imposed upon individual cells are successfully challenged, and removed, cancer can develop. Through the "misapplication" of normal developmental and homeostatic biologic mechanisms cancer cells strive to "survive" without consideration for the rest of the organism.

Cancer is almost as old as life itself. While the first description of human cancers come from deciphered Egyptian papyri, dated to 2,500 B.C. (3), cancer can unmistakably be found in the fossil records, dating hundreds of millions of years ago (4). With these timeframes in mind one can imagine an ongoing evolutionary war, where increasing cellular complexity leads to new developmental and homeostatic pathways, that not only help an organism survive but also provide new pathways, to a damaged individual cell, to successfully transform into cancer. The organism must then evolve new constrains to control cellular behavior and prevent cancer development. Fundamental biological systems, such as growth suppression genes (i.e. retinoblastoma and TP53 genes), apoptosis, contact inhibition, the TGF-β pathway and telomere based replicative mortality, have evolved to be, at least partly, responsible for cancer control. One additional system, whose initial purpose may or may not have been cancer control, is the immune system.

Tumor immunology: a historical perspective

The role of the immune system in cancer control was not always appreciated (5). In fact, even up to 15 years ago, when Hanahan and Weinberg published their now famous "hallmarks of cancer" paper (6), the immune system was not recognized as such. It would take several more years, and the unquestionable clinical success of immune specific therapies, before "*avoiding immune destruction*" would be widely recognized as one of the fundamental hallmarks of cancer (7). However, evidence for the strong relationship between cancer and the immune system dates back to centuries. For example, spontaneous regression of cancers, almost always following severe infections, have been described since at least the 18th century (8), while Virchow microscopically observed immune cell infiltration in tumors as early as 1863 (9). In the 1890's Coley went as far as to inoculate cancer patients with his so called "Coley's toxin", a mixture a killed bacteria species, and was able to reproduce durable clinical responses (10). Ironically, Coley attributed the success of his therapy to "competition" of the streptococcus bacteria

with the parasitic organisms responsible for the cancer, since the infectious etiology of cancer still prevailed scientific thought at the time. The inconsistent success of "Coley's toxin" in the subsequent years, the occasional severe toxicity, and the acquired clonal deletion theory of Burnet (11), according to which tumor are "self" and thus cannot be recognized and attacked by the immune system, led the scientific community away from this promising cancer therapeutic strategy.

In a stunning reversal of opinion however, Burnet and Thomas proposed their cancer immunosurveillance hypothesis in 1957 (12, 13). At about the same time it was recognized that animals could be successfully immunized against transplantable tumors (14) and immunosuppression was linked to cancer (15). In the 1980's the first tumor associated antigens were discovered (16). In the 1990's peripheral T-cell tolerance, preventing tumor-specific immunity, was demonstrated (17). Gradually the first therapeutic immune targets, namely molecules and immune cell subtypes that limit effective anti-tumor activation of the immune system, were recognized and explored (18).

Tumor associated immunosuppressive mechanisms in cancer

A developing cancer attracts the attention of the immune system. Immune cells then infiltrate the cancer site. However, by the time a cancer becomes clinically apparent, it is able to induce local and systemic immunosuppression, which in turn allows the cancer to evade the immune response (18). There are several mechanisms for this to happen. Cancer cells secrete immunosuppressive cytokines, such as TGF-β and IL-10, which shift the nature of the immune cells in the tumor microenvironment from an anti-tumor, or Th1, state to a pro-tumor, or Th2 state (18). Effector T-cells become inactivated and instead the function of regulatory T-cells, which are immunosuppressive in nature, is promoted (19, 20). In addition, tumoricidal macrophages, such as M1 type macrophages, transform into M2 type macrophages, further promoting immunosuppression (21). Recently recognized myeloid derived suppressor cells (MDCS), found in the tumor microenvironment as well as in the circulation, also aid in the process of cancer induced immunosuppression (22). In this type of immunosuppressive environment NK and NKT cell activation is also inhibited (23, 24). As a result, despite the fact that cancer cells downregulate MHC class I expression, they are not targeted by NK cells. Downregulation of tumor associated antigens may be another mechanism of immune system evasion in cancer (25).

Another way cancer cells evade the immune system is by upregulation of immune inhibitory molecules that directly inhibit effector T-cells at the cancer site. Several members of the B7 immunoglobulin superfamily are such inhibitory molecules (26). Upregulation of B7-H1 (PD-L1), B7-H3, B7-H4, B7-H5 and B7-H7 have been shown to inhibit

T-cell activation in various cancers. The expression of Galectin-9, one of the molecules belonging to the galectin family of glycoproteins, also inhibits effector T-cells as well as promotes the function of T-regulatory cells (27-29). HVEM, a molecular switch with dual stimulatory and inhibitory function, can also be expressed by cancer cells, and the inhibitory function can be dominant in tumors because of selective expression of its inhibitory receptor on tumor-infiltrating T-cells (30, 31). Expression of the enzyme IDO in cancer cells inhibits T-cells by breaking down tryptophan, an essential amino acid for T-cell survival, and by generating metabolites that inhibit T-cell function (32). Finally, non-classical MHC type molecules, such as HLA-G, are also known to be expressed by cancer cells and inhibit T-cells (33, 34).

The above immunosuppressive mechanisms re-inforce each other (23, 35) and create an immune environment ideal for tumor growth. While these mechanisms are reviewed in more detail in chapter 2 of the current thesis, with a specific focus on pancreatic cancer, the principles are similar for all malignancies. Current immunotherapeutic strategies aim at reversal of these immunosuppressive mechanisms.

Overcoming the limitations: Immunotherapeutic strategies

Various immunotherapeutic strategies are currently being employed against cancer aiming at re-invigoration anti-tumor immunity (36). While Coley was somewhat successful in achieving that goal back in the 19th century (10), his method, causing erysipelas infections to patients, may not be suitable for our times. Vaccination strategies using tumor associated antigens aim at re-training the immune system to recognize cancer cells, similar to the way it can be trained to recognize diphtheria or measles. However, the analogy may be simplistic, since simple inoculation with tumor associated antigens is not enough, due to local immunosuppressive mechanisms in the tumor microenvironment. The fact, for example, that the first modern immunotherapy to be approved against cancer was a vaccination strategy against prostate cancer (37) comes in sharp contrast with the fact that many large recent vaccination trials have had negative results (38). However, cancer vaccines are improving in sophistication and complexity (39), with attention focused on many aspects such as vaccine adjuvants (40), combination strategies (39) and novel antigen formats (41). With currently hundreds ongoing vaccination clinical trials worldwide, success cannot be far. Adoptive cell transfer therapy, the collection and *ex vivo* manipulation and expansion of autologous T-cells, with the purpose of returning them to the patient with cancer, has been associated with remarkable anti-tumor clinical responses (42). While it may also become an established treatment strategy in the near future, the need to overcome logistic complexities, as well as the short half-life of the infused cells, has kept this otherwise remarkable strategy from being clinically available for the time being (42).

Of all the immunotherapeutic strategies, immune checkpoint inhibitors have been the most successful to date (43-47). First in melanoma and now in lung cancer, renal cell carcinoma and bladder cancer, these therapies are rapidly transforming the overall oncologic treatment of cancer. These antibodies directly interrupt inhibitory interaction between co-inhibitory receptors expressed on T-cells (i.e. CTLA-4, PD-1) and their ligands (members of the B7 immunoglobulin family) expressed on cancer cells and/or tumor-infiltrating leukocytes. Clinical trials are ongoing, not only with the currently approved antibodies but also with new antibodies and combination treatments (48). Agonistic antibodies that target co-stimulatory molecules that directly activate T-cells are also in development (48). The above immunotherapeutic strategies are reviewed in detail in chapter 2 of the current thesis, with a focus on pancreatic cancer. However, similar strategies are currently being employed for HCC (49) and colorectal cancer (50).

Need for immune biomarker development

While the above types of immunotherapy are welcome additions in the war against cancer they are not without pitfalls. The nonspecific activation of the immune system can lead to severe, and occasionally lethal, side effects (51-53). Amongst others, colitis and pneumonitis are well characterized complications. In addition, while there is all the reason for optimism, long term cures are still uncommon. In fact many patients have no clinical benefit from immune checkpoint inhibitors whatsoever. Differentiating patients that will probably benefit from patients that are unlikely to benefit from immunotherapy is of the paramount importance. Such biomarkers would allow patients and doctors to make more informed decisions, such as sparing patients from toxicities associated with the use of these agents. In addition, they would help clinical trials to properly enrich patient cohorts with the right patients and help lower the enormous costs of these compounds. Patients likely to benefit from immunotherapeutics would enter immunotherapeutic clinical trials, while patient unlikely to benefit would enter other types of trials. The problem currently is that such biomarkers do not exist. PD-L1 expression assays, while associated with patient clinical responsiveness to therapies targeting the PD-1/PD-L1 axis, are currently poor biomarkers due to lack of standardization and poor negative predictive value. Thus immune specific biomarkers are urgently needed (54).

Aims of the thesis

In the current thesis we aim to examine the expression of multiple immune related molecules in gastrointestinal cancers with the goal of identifying possible suitable targets for immunotherapy as well as propose promising immune specific biomarkers. We focus on hepatocellular carcinoma, colorectal cancer and pancreatic cancer. With pancreatic cancer we also include patients with ampulary cancer. We focus on the immunohistochemical determination of expression of biomarkers in tumor tissues, as well

as circulating biomarkers, since they have the best chance of clinical translation. Since PD-L1, by itself, appears to be a poor biomarker, we study the expression of multiple immune inhibitory molecules, as well as tumor infiltrating lymphocytes, in conjunction with PD-L1. We hope that the context provided will improve the performance of the proposed biomarkers.

We focus on expression of molecules by cancer cells that are ligands for *co-inhibitory* receptors expressed by T-cells. This means that their receptors are not studied in the current thesis due to the fact that they are expressed exclusively by lymphocytes. In addition, *co-stimulatory* ligand-receptor pairs, such as 4-1BB and OX-40 and their ligands, while promising for cancer immunotherapy, are not studied. To limit the number of immune inhibitory molecules examined at this stage, we focus on molecules of which the biology is relatively well understood (for example a respected receptor is identified on T-cells). This means these molecules can, in principle, become targets for immunotherapy in the near future. In addition, we focus on molecules for which reliable antibodies for immunohistochemical detection in paraffin-embedded formalin-fixed tissue are available. The above criteria have limited our work on the following molecules, which at first sight might appear randomly put together: PD-L1, Galectin-9, HVEM, IDO and HLA-G, in addition to evaluation of tumor infiltrating $CD8^+$ and $FoxP3^+$ lymphocytes.

Finally, in a related activity, we aim to examine the expression of tumor associated antigens in hepatocellular carcinoma. The goal of developing vaccination strategies against HCC requires identification of proper tumor antigens. However, since tumors can downregulate tumor associated antigens in order to evade the immune system, we also look on the possible prognostic role or tumor associated antigen expression in HCC.

Outline of the thesis

In chapter 1 we give a general introduction on the importance of the immune system in cancer and the rationale behind the work of this thesis. In chapter 2 we continue our introduction by focusing, in more detail, on immune inhibitory mechanisms, as well as experimental immunotherapies, in pancreatic cancer. In chapters 3 and 4 we use tissue microarrays to investigate the expression and potential prognostic value of the inhibitory molecules PD-L1, Galectin-9, HVEM, IDO, HLA-G and $CD8^+$ TILs and $FoxP3^+$ TILs in hepatocellular carcinoma, pancreatic cancer and ampullary cancer. In chapter 5 we move beyond tissue microarrays and examine the importance of $CD8^+$ and $FoxP3^+$ TILs in colorectal cancer liver metastasis, using full tissue slides. This allows us to comment on the importance of TIL location in the tumors. In chapters 6 and 7 we move beyond cancer cell expression of biomarkers and discuss circulating biomarkers. In chapter 6 we discuss what is known about routine immune laboratory tests and their association

to cancer survival. In chapter 7 we investigate the prognostic value of circulating PD-L1 and Galectin-9 in patients with hepatocellular carcinoma. In chapter 8 we investigate expression of tumor associated antigens as possible targets for vaccination in hepatocellular carcinoma, as well as comment on the prognostic role of tumor associated antigen expression. In chapter 9 we provide a general discussion of our thesis. We focus on issues that require more discussion, such as providing additional hypothesis that explain our findings. We also discuss technical aspects of our thesis, as well as ideas for future research, with the goal of successfully developing clinically useful, immune specific, biomarkers for the era of cancer immunotherapy.

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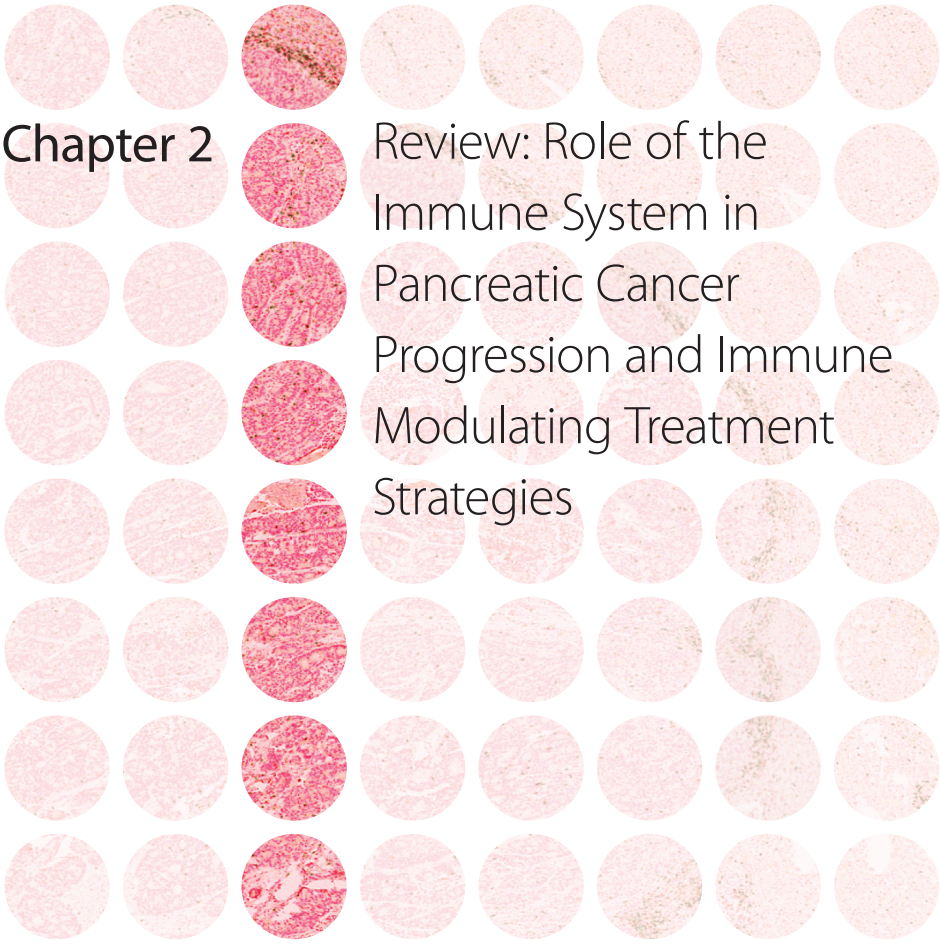

Chapter 2

Review: Role of the Immune System in Pancreatic Cancer Progression and Immune Modulating Treatment Strategies

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ABSTRACT

Traditional chemotherapeutics have largely failed to date to produce significant improvements in pancreatic cancer survival. One of the reasons for the resilience of pancreatic cancer towards intensive treatment is that the cancer is capable of high jacking the immune system: during disease progression the immune system is converted from a system that attacks tumor cells into a support structure for the cancer, exerting trophic actions on the cancer cells. This turn-around of immune system action is achieved through mobilization and activation of regulatory T cells, myeloid derived suppressor cells, tumor-associated macrophages and fibroblasts, all of which suppress CD8 T cells and NK cells. This immune suppression occurs both through the expression of tolerance-inducing cell surface molecules, such as PD-L1, as well as through the production of "tolerogenic" cytokines, such as IL-10 and TGF-b. Based on the accumulating insight into the importance of the immune system for the outcome of pancreatic cancer patients multiple new immunotherapeutic approaches against pancreatic cancer are being currently tested in clinical trials. In this review we give an overview of both the immune escaping mechanisms of pancreatic cancer as well as the new immune related therapeutic strategies currently being tested in Pancreatic cancer clinical trials.

INTRODUCTION

Pancreatic cancer is the 5th leading cause of cancer related death in the developed world with more than 260,000 deaths annually worldwide [1]. Due to its aggressive nature and late presentation 5-year survival is a dismal 6%. Research efforts have mainly focused on improvements in surgical technique, radiation therapy and chemotherapeutics. However, advancements in traditional chemotherapeutics have been especially slow, and despite the recent success of the FOLFIRINOX regiment in metastatic disease long term significant benefit has not materialized.

Recently, research efforts have focused on the role of the immune system in the development and progression of cancer. It is now known that both the innate and the adaptive immune system are active against human cancers [2]. Effective anticancer function of the immune system requires cytotoxic CD8 T cells, T helper-1 (Th1) cells, mature dendritic cells (DCs), activated pro-inflammatory macrophages (M1) and NK cells. However, cancer cells induce both local and systemic immune dysfunction thus avoiding detection by the immune system [3]. Under the tumor induced immunosuppressive environment T helper cells acquire a T helper cell type 2 phenotype (Th2), which does not support cytotoxic CD8 T cell responses and is tolerant toward tumors, macrophages

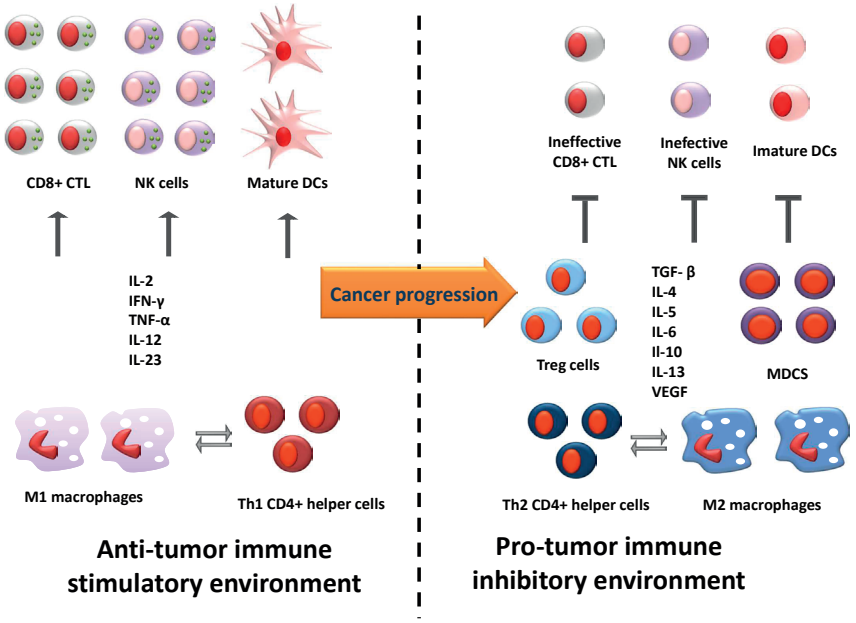

Figure 1: Changes in the tumor immune microenvironment during cancer progression.

switch to the immunosuppressive M2 state, while T regulatory cells (Tregs) and myeloid derived suppression cells (MDCS) inhibit effector immune responses (Fig. 1).

Tumor immune modulation and evasion starts at the level of the cancer cell. Cancer cells use at least three mechanisms to modulate the immune system and avoid detection by effector immune cells: contact dependent factors (expression of immune system check-point ligands such as PD-L1), secretion of soluble immunosuppressive factors (such as IL-10, TGF-b and VEGF) and interference with MHC class I peptide presentation (through down-regulation of MHC class I expression or disabling of the antigen degradation or antigen insertion into the MHC class I grove). Despite the fact that downregulation of MHC class I makes cancer cells the target of NK cells, cancer cells influence the cytotoxic activity, the presence of activating receptors, and the numbers and proliferation of NK cells, thus further avoiding detection and destruction. Through these mechanisms cancer cells have a profound local and systemic immunomodulating effect which leads to general immunosuppression and tumor progression.

It is safe to predict that immune modulation strategies in pancreatic cancer will be widely explored in the years to come in view of the increasing scientific knowledge in the field, the success of immunotherapeutic strategies in other cancers, and the evident inadequacy of competing treatment modalities. In this review we describe the immune escape mechanisms of cancer, with a primary focus on pancreatic cancer, and we discuss immune modulating treatment strategies tested in pancreatic cancer clinical trials. We focus primarily on recent scientific insights, as well as clinical trials that are characteristic of the given treatment strategies.

In pancreatic cancer a dysfunctional immune system aids rather than controls cancer

Immune cells in pancreatic cancer promote an immunosuppressive and anti-inflammatory environment (Fig. 1)

T regulatory cells.

Of all the different types immune cells, Tregs have gotten the most attention in tumor immunology research. They are generally defined as CD4+CD25+FoxP3+ cells and they are found in the tumor microenvironment at increased numbers. By expression of CTLA-4 and secretion of IL-10 and TGF-b, among others, Tregs suppress exaggerated immune responses and are essential in the prevention of auto-immune diseases. In cancer however, they produce a local immunosuppressive environment ideal for tumor growth [4,5]. Patients with pancreatic cancer have increased numbers of Tregs both in

the circulation and at the tumor site. Moreover, the presence of Tregs at the tumor site correlates with more advanced presentation of disease [6,7], a lower chance of surgical resection and a worse survival after resection [8], while low Treg percentage in the circulation one year post resection correlates with improved survival [8]. In addition, as levels of Treg cells increase, levels of the CD8+ effector cells decrease [7]. Hence the Treg compartment represents an attractive target in pancreatic cancer.

Myeloid derived suppressor cells

MDSCs are immature myeloid cells that suppress both innate and adaptive immunity [9]. Factors contributing to their action in immunity include sequestration of cysteine (an essential amino acid for T cell activation), expression of high levels of arginase (resulting in depletion of L-arginine which is required by T cells for protein synthesis), increased production of reactive oxygen species, impairment of T cell homing to lymph nodes and secretion of TGF-b. These factors inhibit the function of effector T cells and NK cells and promote the development of Tregs. In a mouse model of spontaneous pancreatic cancer development the extent of immune suppression induced by MDSCs increased during the progression from premalignant lesions to pancreatic cancer [10]. Patients with pancreatic cancer have increased MDSCs in the circulation compared to healthy controls, and MDSCs levels correlate with levels of the Th2 cytokine IL-13 and Treg cell numbers [11]. Increased levels of circulating MDSC is an independent poor prognostic factor in patients with pancreatic cancer [11].

Tumor associated macrophages (TAMs)

TAM tumor infiltration is associated with worse prognosis in multiple cancers. Macrophages, due to stimuli from the tumor microenvironment such as IL-10, TGF-b and other cytokines, switch their differentiation from M1 (pro-inflammatory or classically activated macrophages) to M2 (anti-inflammatory or alternatively activated macrophages) which have pro-tumor properties, such as promotion of angiogenesis, matrix remodeling and tumor metastasis, as well as suppression of adaptive immunity [12,13]. TAMs interact with the immune system by multiple mechanisms such as through secretion of IL-10 and TGF-b or by expression of immune inhibitory ligands such as PD-L1. In pancreatic cancer TAMs are significantly increased in tumor tissue [14]. In addition, the presence of M2 polarized TAMs is associated with worse prognosis in pancreatic cancer [15].

Dysfunctional immune effector cells in pancreatic cancer

Cytotoxic and helper T cells

In general both the number and function of cytotoxic and helper T cells is known to be affected in various cancers. In general, the presence of increased numbers of cytotoxic T

cells in the tumor is associated with a better prognosis while both cytotoxic and helper T cells are functionally impaired under the influence of immunosuppressive cytokines, leading to predominately Th2 (tumor tolerating) rather than Th1 (tumor killing) responses. In patients with pancreatic cancer circulating functional tumor-reactive CD8 T cells can be detected both in the circulation and in the bone marrow [16], while the presence of both CD8 and CD4 T cells in the tumor is correlated with better prognosis [17]. In addition when pancreatic lesions progress from premalignant to malignant, CD8+ effector cells decrease in number while the presence of Treg cells are increased [7]. At the same time both circulating CD8+ cytotoxic T cells and CD4 T cells from patients with pancreatic cancer have impaired function while Th2, rather than Th1, responses predominate [18,19].

Dendritic cells (DC)

Dendritic cells play a critical role in the anti-tumor response. They belong together with macrophages to a class of cells called antigen-presenting cells (APC), which in contrast to all other cells in our body, express MHC class II and can therefore present antigenic peptides to CD4 T cells. By virtue of their efficient machinery to internalize (tumor) antigens, degrade them into peptides and present them on both MHC class I and II molecules to CD4 and CD8 T cells respectively, they are the most professional APCs. They can prime tumor specific effector T cells to start attacking cancer cells. However, due to the immunosuppressive tumor microenvironment their maturation and survival in cancer is significantly impaired. In pancreatic cancer patients while the presence of DCs in the circulation, or in the tumor tissue, is associated with prolonged survival [20], DCs also display maturation defects [21], suggesting that therapy aimed at improving DC functionality could be beneficial.

NK cells

Through MHC class I loss, which is a common event in pancreatic cancer [22], pancreatic cancer cells become the target of NK cells. However, pancreatic cancer cells can escape control by this system. Indeed, NK cell activity is diminished in patients with pancreatic cancer [23]. Activating receptors, such as NKG2D, which are necessary for the activation of NK cells, are reduced on the surface of NK cells in patients with pancreatic cancer and reduced levels are associated with advanced disease [24]. On the other hand higher absolute levels of NK cells in the circulation are associated with improved survival [25], indicating that the immune system, through NK cells, still exerts control on cancer growth despite disease progression.

Contact dependent mechanisms of immune modulation: expression of co-stimulatory and co-inhibitory immune ligands and receptors by pancreatic cancer cells

T cells are activated by a complex interaction of ligands and receptors (Fig. 2). Specifically, APCs present antigenic peptides on both MHC class I (to stimulate CD8 T cells) and II (to activate CD4 T helper cells) molecules to the T cell receptor (TCR) on the surface of T cells. A complex balance of multiple stimulatory and inhibitory receptors on the surface of T cells ensures the proper function of the immune system. Binding of the MHC molecule to the TCR is not sufficient to initiate activation of the T cell. Activation requires additional ligand binding to co-stimulatory receptors such as CD28, CD40, OX40, and 4-1BB. Activated CD4 T cells express CD40-ligand, which activates APC via ligation of CD40, thus forming a stimulatory loop between APC and T cells. On the contrary, receptors such as CTLA-4 and programmed death 1 (PD-1) expressed on the surface of activated T cells inhibit T cell activation upon binding to their ligands CD80/CD86 and PD-L1/ PD-L2 respectively. In cancer this mechanism of immune co-stimulation and co-inhibition has been found to be extremely important [26].

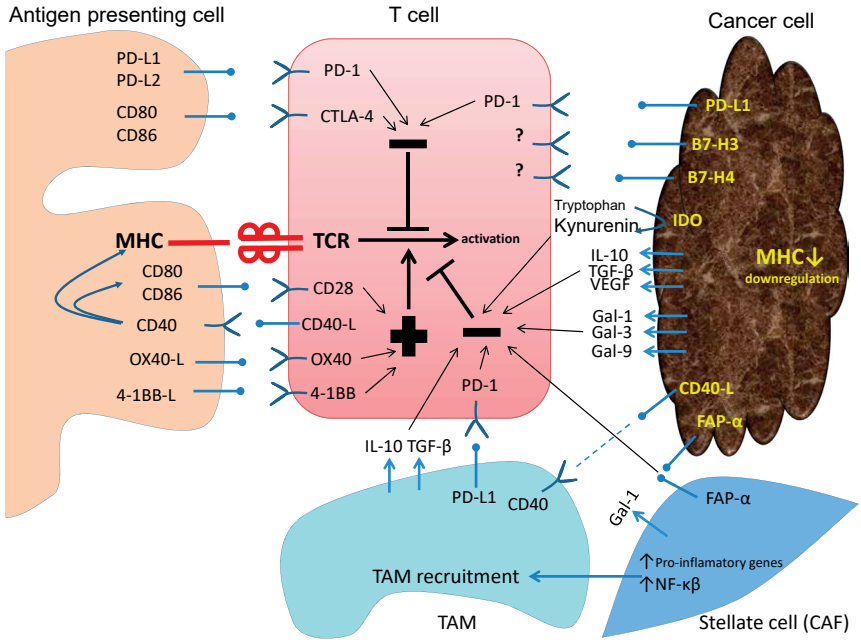

Figure 2: Immune co-stimulatory and co-inhibitory ligands and receptors and soluble immune modulating factors involved in T-cell activation and inhibition.

Pancreatic cancer cells express a number of ligands that are meant to inactivate cytotoxic T-cells in the local tumor microenvironment. For example the ligand for PD-1 (PD-L1) is

expressed by pancreatic cancer cells and its expression is associated with reduced cytotoxic T-cell infiltration, advanced stage of disease and poor prognosis [27,28]. Pancreatic cancer cells express both CD40 and CD40L resulting in the secretion of several pro- and anti-inflammatory cytokines in the tumor microenvironment [29]. High expression of CD40L on tumor cells has been associated with good prognosis in pancreatic cancer patients [29]. Other immune inhibitory ligands with a role in pancreatic cancer are B7-H3 and B7-H4, the receptors of which are still unknown. The expression, for example, of the immune inhibitory ligand B7-H3 has been associated with better prognosis in pancreatic cancer patients [30], while blockade of the B7-H3 ligand interaction leads to tumor shrinkage in animal models [31]. Even direct expression of FoxP3 by pancreatic cancer cells, mimicking thus Treg cells, has been detected [32]. Thus the pancreatic cancer cell is clearly under selection pressure to express immunosuppressive molecules providing hope that targeting this escape mechanism can be therapeutically meaningful.

Direct secretion of soluble immunosuppressive factors by pancreatic cancer cells (Fig. 2)

Direct secretion of immunosuppressive factors by pancreatic cancer cells is another mechanism of escaping the immune system. TGF-b induces tumors to secrete VEGF and matrix metaloprotein-2 which are associated with advanced stage of disease and metastasis [33,34]. Tumor derived TGF-b and IL-10 inhibit the development of Th1 responses whereas they promote Th2 responses [19]. Secretion of multiple cytokines by pancreatic cancer cells contribute to the general immunosuppressive micro-environment of pancreatic cancer by switching the balance from a Th1 to a Th2 state [35].

Indoleamine 2,3-dioxygenase (IDO) is an enzyme upregulated in pancreatic cancer cells and catabolizes tryptophan into kynurenine. Depletion of tryptophan, as well as secretion and accumulation of kynurenine in the tumor microenvironment, inhibits T cell activation and stimulates Treg differentiation [36]. In pancreatic cancer expression of IDO attracts Tregs to the tumor microenvironment [37]. Inhibitors of IDO are already under phase I investigation.

Galectins are soluble immunomodulating glycoproteins that are involved in T-cell homeostasis, preservation of fetal-maternal tolerance and suppression of autoimmunity. In cancer galectins have been shown to contribute to the immunosuppressive tumor microenvironment and evasion of immune responses. The best studied galectins in cancer immunomodulation are Gal-1, Gal-3, and Gal-9. Gal-1, is known to promote a Th2 cytokine profile in cancer, induce IL-10 production in Tregs and is important in immune cell trafficking and DC physiology [38]. In pancreatic cancer Gal-1 is overexpressed by tumor cells [39] and has been identified as a proteomic biomarker highly correlated with stage of disease [40]. Gal-1 is also expressed on stellate cells and contributes to

stellate cell activation [41] and maintenance of the immunosuppressive microenvironment [42]. "At the same time, Gal-1 is found underexpressed on the stromal tissues of long term pancreatic cancer survivors [43], indicating that the prognostic significance of Gal-1 expression is promising'. Blocking molecules for Gal-1 have entered phase I clinical trials, but not yet in pancreatic cancer. While Gal-9, partly through its complex interaction with TIM-3, modulates T cell, NK cell and MDSC activity [44], it's precise role in pancreatic cancer has not yet been investigated. Another galectin with an important role in immune modulator in cancer is Gal-3 [45]. Gal-3 is overexpressed in pancreatic cancer cells, secreted in the serum of pancreatic cancer patients and is associated with tumor differentiation [46,47]. However, not much is known about the role of Gal-3 as an immune modulator in pancreatic cancer.

Cancer associated fibroblasts (CAFs) and the immune system

The immune infiltrate of pancreatic cancer is but a part of the tumor microenvironment. Cells of mesenchymal origin (tumor associated fibroblasts), associated with extra cellular matrix proteins and tumor associated vasculature, are other important components. A dense desmoplastic stroma reaction has long been recognized as a hallmark of pancreatic cancer and it is known to actively promote tumorigenesis and resistance to therapy. CAFs, however, also interact with the immune system in ways that promote tumor progression (Fig. 2). CAFs attract TAMs to the tumor microenvironment, though an NF-jb related mechanism, resulting in increased fibrosis and tumor growth [48]. In addition CAFs secrete fibroblast activation protein (FAP-a) which further suppresses effector T cells through interfering with TNF-a and IFN-c related activation [49]. FAP-a is found overexpressed on both the pancreatic cancer stroma and on pancreatic cancer cells [50] while anti-FAP-a monoclonal antibodies are currently in clinical development. Another treatment strategy that affects both the immune system (TAMs) and the CAFs is the use of CD40 agonists, to be discussed later.

Treatment strategies based on the interaction of pancreatic cancer with the immune system

Several immunotherapeutic strategies are being actively tested in clinical trials. We will discuss strategies aimed at antigenic stimulation of T cells (vaccination), increasing the number of tumor specific cytotoxic T cells (adoptive cell transfer), interfering with co-stimulatory and inhibitory receptors and ligands and depleting Treg (Fig. 3). An overview of the clinical trials is provided in Table 1.

Vaccination strategies

Tumor antigens are expressed by a significant portion of pancreatic tumors. They can be normal proteins, present on normal tissues, albeit at much higher concentrations

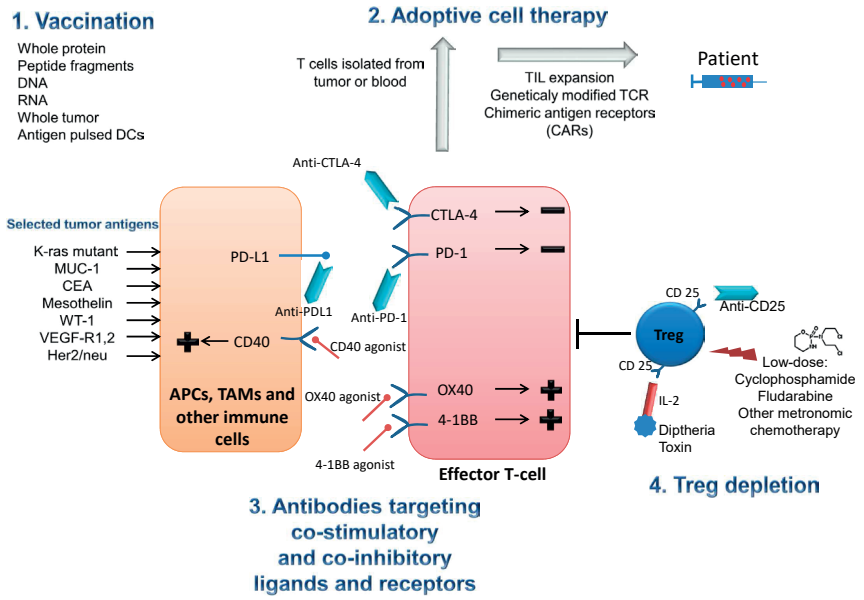

Figure 3: Diagrammatic representation of immune treatment strategies under investigation in pancreatic cancer.

on cancer cells, such as MUC-1, CEA, WT1, VEGF-R and mesothelin, or mutated proteins, present exclusively on pancreatic cancer cells, such as mutated K-ras. They can be administered in various formats such as whole proteins, peptide fragments, DNA or RNA (Fig. 3). In animal models, administration of protein and peptide vaccines can cause antigen specific T-cell responses that lead to tumor eradication. In humans, antigen specific T-cell responses are also commonly observed, although actual tumor responses are less common.

One of the most promising tumor antigens is mutated K-ras, which is uniquely expressed in pancreatic cancer cells. In one clinical study vaccination with mutated K-ras resulted in 20% long term survivors [51]. The majority of survivors expressed antigen specific T cell responses, while none of the patients that progressed demonstrated such response. Although not all studies have been equally promising [52], a recent randomized phase-II study suggested a 2.8 months improvement in overall survival in patients receiving a recombinant mutated K-ras vaccine [53]. A randomized phase II placebo controlled trial using recombinant mutated K-ras protein for vaccination in combination with gemcitabine, in patients with resected pancreatic cancer, is currently ongoing [54].

Table 1 Selected immunotherapy related clinical trials in pancreatic cancer with an emphasis on recent and larger studies. Smaller studies are included when they represent an example of an immunotherapy strategy not otherwise tested in pancreatic cancer.

Phase	Types and number of pts	Type of antigen(s)	Name of antigens	Adjuvant or concomitant therapy	Results	Ref.
Vaccination trials						
R - II	176 Ras mutant positive pts post resection	Recombinant protein	Mutated K-ras	Yeast, Gemcitabine	2.4mo improvement in OS	**[53]**
I/II	10 surgically resected pts and 38 pts with advanced disease	Recombinant protein	Mutated K-ras	GM-CSF	Peptide-specific immunity induced in 58% of pts and responders had increased MS (148 vs 61 days). 20% long term survivors.	**[51]**
R-II	100 pts following resection	Recombinant protein	Mutated K-ras	Gemcitabine	Ongoing	**[54]**
R - III	255 metastatic pts following gemcitabine failure	Gene expressing viral vector	MUC-1, CEA	TRICOM, GM-CSF	No OS benefit	**[56]**
I/II	12 pts with surgically resected pancreatic (10 pts) or biliary (2pts) cancer	Antigen pulsed DCs	MUC-1	None	4 out of 12 pts alive at 4 years	**[70]**
R-II/III	150 advanced unresectable pts	Peptide	VEGF-R2	Gemcitabine	Completed, not reported yet	**[60]**
I/II	17 unresectable, recurrent or metastatic pts	Peptide	VEGF-R1 VEGF-R2	Gemcitabine	Completed, not reported yet	**[59]**
R-II	Unresectable, recurent pts	Peptide	WT-1	Montanide ISA51 (synthetic peptides derived from malaria proteins), Gemcitabine	Ongoing	**[57]**
I/II	48 pts with non-resectable disease	Peptide (only peptide is not ATC!. Or did they transfer cells, e.g. DC with peptides?	Telomerase	GM-CSF	24/38 with immune responses. Induction of immune response correlated with improved survival	**[61]**
R-III	178 chemotherapy naïve, advanced PC pts.	Peptide (same question)	Telomerase	Gemcitabine	No overall survival benefit	**[62]**

Table 1 (continued)

Phase	Types and number of pts	Type of antigen(s)	Name of antigens	Adjuvant or concomitant therapy	Results	Ref.
I	12 pts with Her2/neu overexpressing tumors, including PC	Genetically modified viral vector expressing antigenic peptide	Her2/neu	None	Ongoing	**[63]**
II	60 pts with resected disease	Lethally irradiated, genetically engineered allogeneic whole tumor	Whole tumor	GM-CSF secreting allogeneic PC cells , 5FU chemoradiation	No OS benefit compared to historical control (HR: 0.96, 95% CI, 0.68–1.35, P = 0.8). Mesothelin-specific CD8+ T responses correlated with DFS	**[65]**
II	60 pts with resected disease	Lethally irradiated, genetically engineered allogeneic whole tumor	Whole tumor	GM-CSF secreting allogeneic PC cells, cyclophosphamide and cetuximab	Completed, not reported yet	**[66]**
R-II	60 pts with resected disease	Lethally irradiated, genetically engineered allogeneic whole tumor	Whole tumor	GM-CSF secreting allogeneic PC cells and IV vs oral metronomic cyclophosphamide	Ongoing	**[68]**
I	12 pts with resected disease	Lethally irradiated, genetically engineered allogeneic whole tumor	Whole tumor	GM-CSF secreting allogeneic PC cells and cyclophosphamide followed by localized radiation (SBRT) and FOLFIRINOX	Ongoing	**[69]**
II	56 pts with resected disease	Lethally irradiated genetically engineered allogeneic whole tumor	Whole tumor	GM-CSF secreting allogeneic PC cells	Ongoing	**[67]**
R - II	90 pts with metastatic disease	Lethally irradiated genetically engineered allogeneic whole tumor and listeria	Mesothelin and whole tumor	GM-CSF secreting allogeneic PC cells, cyclophosphamide	Ongoing	**[58]**
I	14 pts with resected disease	Attenuated Listeria vector	Mesothelin and whole tumor	Adjuvant chemotherapy and radiation	3 out of 14 patients developed delayed type hypersensitivity and DTH was associated with prolonged survival	**[64]**

Table 1 (continued)

Phase	Types and number of pts	Type of antigen(s)	Name of antigens	Adjuvant or concomitant therapy	Results	Ref.
Adoptive cell therapy trials						
I/II	8 Pts with unresectable and 20 with resectable PC	CTLs expanded from PBMCs and exposed to MUC-1 expressing PC cell line	MUC-1	None	Unresectable pts mOS 5 months. Resectable pts 3 years survival 19%. Only 1 unresectable pt presented with hepatic metastasis	**[80]**
I	20 pts with unresectable or metastatic disease	Antigen pulsed DCs and cytotoxic T lymphocytes	MUC-1	None	1 patient had complete response and 5 patients stable disease	**[79]**
II	48 Pts with various metastatic cancers likely to include PC pts	TCR genetically engineered peripheral T cells	CEA	IL-2	Ongoing	**[81]**
I/II	136 mesothelin expressing cancer pts (including PC pts) with metastatic disease	CAR engineered lymphocytes	Mesothelin	Fludarabine, cyclophosphamide, IL-2	Ongoing	**[82]**
Co-stimulatory and co-inhibitory receptors and ligand antibody trials						
I	30 locally advanced, unresectable or metastatic pts	CTLA-4	Ipilimumab	PC vaccine (GM-CSF secreting allogeneic PC cells)	Ongoing	**[86]**
I	37 metastatic pts	CTLA-4	Tremeli-mumab	Gemcitabine	Ongoing	**[87]**
II	27 pts with unresectable or metastatic disease	CTLA-4	Ipilimumab	None	1/27 pts experienced a delayed and durable response	**[85]**
I	28 metastatic pts	CTLA-4	Iplimumab	Gemcitabine	Ongoing	**[88]**
II	29 resected pts	PD-1	CT-011	Gemcitabine	Not recruiting yet	**[94]**
I	21 pts with surgically incurable PC	CD40	CP-870,893	Gemcitabine	4 out of 21 pts had partial response, PFS and OS favorable compared to historical controls	**[96]**
I	10 pts with resectable PC	CD40	CP-870,893	Gemcitabine	Ongoing	**[97]**

MUC-1 is a membrane bound glycoprotein known to promote pancreatic cancer epithelial to mesenchymal transition and invasiveness. It also induces CD8 T cell responses and the production of anti-MUC antibodies is associated with improved survival [55]. However, in a large randomized phase-III clinical trial of 255 patients vaccination with a MUC-1 and CEA expressing viral vector showed no overall survival benefit [56].

A phase II vaccination trial in pancreatic cancer using WT-1 as antigen is ongoing [57], while a mesothelin vaccine, using genetically modified live attenuated listeria as a vector for the antigen, has also entered clinical trials [58]. Two peptide vaccine studies using VEGF-R1 and VEGF-R2 are completed but have not reported results yet [59,60]. Telomerase reverse transcriptase (hTERT) is a highly immunogenic antigen and has been the target in several vaccination studies. A small phase I/II study in patients with pancreatic cancer showed T cell responses in 63% of vaccinated patients, and prolonged survival in patients exhibiting T cell responses [61]. However, a large phase III study failed to show a survival benefit in pancreatic cancer [62]. Finally vaccination against Her2/neu is also being tested in a phase I study in patients with pancreatic cancer [63].

Whole tumor cell vaccines are another strategy that has shown promise in pancreatic cancer. The advantage of using whole tumor vaccines is that the vaccine contains all possible tumor antigens and can be patient specific. These are more challenging to develop because of the difficulty of obtaining sufficient numbers of tumor cells from pancreatic cancer patients and the time it takes to process the tumor cells. To bypass these problems allogeneic tumor cell lines, modified to increase immunogenicity, have been developed to serve as vaccines. In a phase I clinical study, tumor cells, which were modified to express the immunomodulating cytokine GM-CSF, where given to 14 patients [64]. Three patients had delayed-type hypersensitivity responses to autologous tumor cells and those 3 patients had a longer disease free survival. In a subsequent phase-II study with a similar approach, 60 patients with resected pancreatic cancer were treated, yielding a disease free survival of 17 months and an overall survival of 24 months [65]. While the results were not superior to historical controls other studies using similar approaches, or combining whole tumor vaccination with cyclophosphamide alone, or with conventional chemotherapy, are ongoing [66–69].

Antigen pulsed DCs is another vaccination strategy where patient DCs are isolated, pulsed with peptides, autologous, or allogeneic tumor lysate, or transfected with RNA, and injected back to the patients. In a study of 12 patients (10 with pancreatic cancer), where MUC-1 pulsed DCs were given as adjuvant therapy following resection, 4 of the 12 patients were alive at 4 years [70].

One reason for the apparent lack of significant objective responses in human studies may not be the choice of the antigen bur the inability of the antigen to be properly presented by APCs. Intact tumor proteins need to be internalized by APCs and degraded to peptides. They are then presented on MHC class II molecules, thus stimulating CD4 T cells well, but not CD8 T cells. This issue may be overcome by the process known as "cross-presentation" where DCs can, through receptor mediated endocytosis, process glycosylated proteins and present them on MHC class I molecules thus stimulating CD8+ cells. Cross-presentation is an active area of research in tumor immunology. On the other hand tumors antigens can be presented through vaccines as a mix of synthetic peptides that fit the groove of MHC class I molecules, thus bypassing the need for uptake and degradation. These peptides can stimulate CD8 T cells well but not CD4 T cells. Since CD4 T cells are necessary for the effective function of CD8 T cells this may explain the lack of apparent success in many of these trials.

Given the limitations encountered with protein and peptide vaccinations both DNA and RNA have been used to induce antigen presentation in APCs. The genetic material is either injected subcutaneously, or electroporated ex vivo into autologous dendritic cells which are subsequently transferred to the patient. Use of messenger RNA (mRNA) is of particular interest recently since it is not integrated into the genome and therefore RNA vaccination is not regarded as gene therapy. Dendritic cells exposed to mRNA are able to present peptides of the encoded antigen on both MHC class I and class II molecules, and therefore activating both CD4 and CD8 T cells. Major advantages of using mRNA vaccines are that they lead to sustained CD4 and CD8 T cell immunity, are easy to apply, are cost efficient, and are safe in human studies [71]. While these techniques have only been tested in animal models of pancreatic cancer, multiple human clinical studies are ongoing for other cancers.

Adoptive cell transfer (ACT)

In this strategy T-cells are removed from the tumor tissue (TILs), expanded ex-vivo, and "manipulated" before being returned to the host in massive numbers. This allows manipulation of the T-cells, such as priming of the cells to tumor antigens, or transfection with recombinant DNA encoding for T cell receptors specifically directed towards tumor-antigens. Obviously, it is a much more cumbersome technique than vaccination and, at least initially, the half-life of the infused cells was very short for therapeutic benefit, a problem that is now largely overcome.

A typical protocol involves isolation of TILs from original biopsy sites, or metastatic sites, followed by rapid expansion. In the meanwhile, during the rapid expansion process, the patient is receiving chemotherapy, usually low dose cyclophosphamide and/or fluda-

rabine, at doses aiming to promote prolonged lymphodepletion, although anti-CD45 antibodies, total body irradiation or myeloablative chemotherapy can be used in the right clinical setting. The expanded TILs are then re-infused in combination with immune adjuvant therapy such as IL-2. Using this approach results have been very encouraging in melanoma human studies with response rates in selected patients of over 50% [72].

Genetically modified TCRs

Since abundant patient tumor material is not always available genetic modification of autologous isolated peripheral lymphocytes is another option. In these protocols lymphocytes are isolated peripherally (not from the tumor) and then exposed to retroviral vectors encoding for specific genetically modified T cell receptors (TCR) designed to target specific tumor antigens. In melanoma targeting the tumor antigens MART-1 or gp-100 led to response rates of 30% and 19%, respectively but severe uveitis and necrotic skin rashes were a significant problem [73,74]. Targeting tumor antigens that are not expressed in normal tissues, such as the testis associated antigen NY-ESO-1, has led to 45% response rate without noticeable toxicity [75]. Studies in renal cell carcinoma underline the importance of choosing the right tumor antigen and transduction method in order to bypass on-target toxicity and anti-immune reactivity against the transduced T-cells [76].

Chimeric antigen receptors (CARs)

Another related approach is with the use of lymphocytes genetically engineered to carry chimeric antigen receptors (CARs) [77]. The extracellular domain of CARs is an Fab fragment of an antibody that is designed to recognize a specific tumor associated antigen. The intracellular domain of CARs is the signal transducing intracellular domain of the TCR. Thus T-cells target specific tumor antigens and they are activated upon binding. In addition, domains of costimulatory molecules have been engineered into the intracellular domain of CARs in order to further increase T-cell activation upon binding. The advantage of CARs over genetically engineered TCRs is that several different receptors recognizing different tumor antigens and targets, or expressing several co-stimulatory molecules, can be engineered into the T-cells, thus broadening the choice of tumor targets and tissue specificity. Clinical trials using CAR engineered T-cells are ongoing in multiple cancers.

In pancreas cancer results in mouse models using adoptive cell therapy have been successful [78]. Human studies are few but responses have been seen using expanded TILs in combination with MUC-1 pulsed DC's [79]. In a study using CTLs expanded from PBMCs and exposed to a MUC-1 expressing pancreatic cancer cell line only 1 out of 8 unresectable patients developed hepatic metastasis [80]. A study with a genetically

engineered TCR targeting CEA, and likely to include pancreatic cancer patients, is ongoing [81]. A study using anti-mesothelin CAR engineered lymphocytes is also ongoing in mesothelin expressing patients, which will include pancreatic cancer patients [82].

Strategies targeting co-stimulatory and inhibitory receptors and ligands

Various monoclonal antibodies have been developed that either act as inhibitors of the inhibitory receptor-ligand interaction on T-cells and tumors (i.e., CTLA-4/CD-80/CD86, PD-1/PDL1), or act as agonist of co-stimulatory receptors on T-cells (i.e., CD40, OX40, 4-1BB). The suppressive receptor CTLA-4, expressed on Tregs and exhausted CD8 T-cells was the first to be successfully targeted. Ipilimumab is a monoclonal antibody that binds and inhibits CTLA-4. In metastatic melanoma, in previously untreated patients, ipilimumab added to dacarbazine improved overall survival, compared to dacarbazine alone, from 6.4 to 10.1 months [83], while in previously treated patients, ipilimumab alone again improved overall survival from 6.4 to 10 months [84]. Given these impressive results ipilimumab, together with tremelimumab another anti-CTLA-4 antibody, are currently being tested in multiple clinical trials in pancreatic cancer [85–88]. In the first reposted results [85], out of 27 patients with advanced pancreatic cancer who were treated with single agent ipilimumab, only 1 experienced a delayed response, indicating that single agent ipilimumab may not be effective in this disease and combination therapies might be necessary.

Much like antibodies to CTLA-4, antibodies against PD-1, and its ligand (PD-L1), have entered clinical trials with great success producing response rates of 17–38% in advanced melanoma and 10–18% in advanced non-small cell lung cancer [89–91]. Expression of PD-L1 on the cancer cells appears predictive to response to therapy since only patients expressing the ligand responded to anti-PD1 therapy [92]. In the study with the anti-PD-L1 antibody however, from the 14 patients with pancreatic cancer included none responded [89]. Despite this observation, given the small sample size of included pancreatic cancer patients, as well as the recurrent observation that PD-L1 overexpression in pancreatic cancer is associated with advanced disease and poor prognosis [27,93], clinical studies in pancreatic cancer are currently planned [94].

More recently the combination of anti-CTLA-4 and anti-PD1 therapy has been tested in 53 patients with advanced melanoma [95]. The combination produced a response rate of 40% while at the maximal tolerated dose the response rate was 53%. Responses were rapid and durable while toxicity was similar to that seen with monotherapy. Obviously this is another strategy that will be tested in pancreatic cancer should monotherapy with these drugs proves to have insufficient activity.

The combination of CD40 agonist and gemcitabine against pancreatic cancer was associated with some clinical responses in a clinical trial [96], although these tumor responses were difficult to differentiate from responses to gemcitabine alone. However, replication of the study in mice showed that the tumor was infiltrated with tumoricidal CD40 activated macrophages which led to the depletion of the tumor stroma [96]. This process was independent of the presence of gemcitabine, indicating that the CD40 agonists therapeutic effect is through activation of macrophages and antagonizing the effect of CAFs. Further studies are ongoing [97]. Anti-OX40 antibodies and IDO inhibitors are currently undergoing testing in various cancers but not yet in pancreatic cancer.

Strategies to deplete Treg cells

Given the importance of Tregs in attenuating anti-tumor activity by the immune system, one obvious strategy is to target Tregs directly. At least 2 different types of compounds have been developed and tested in clinical trials. In one such strategy denileukin diftitox, an IL-2-diptheria toxin fusion protein that binds to the IL-2 receptor alpha chain (CD25), and is highly expressed on Treg, has been successful in melanoma and renal cell carcinoma clinical trials in partially depleting Tregs and improving vaccine induced cytotoxic T cell responses [98,99]. Significant clinical responses were seen in 5 out of 16 patients in one of the studies [99], including a near complete response. However, not all studies have been encouraging [100]. In another strategy, a monoclonal antibody directed against CD25 has been developed and has shown durable reduction of Treg cell numbers [101].

Back in the 1970's and early 1980's scientists noticed that low dose cyclophosphamide had "chemoimmunologic" effects by depleting "T-suppressor cells" and enhancing delayed type hypersensitivity reactions if given before vaccination. It has now been clearly demonstrated that low dose cyclophosphamide temporarily depletes Tregs, augments T cell responses and is frequently given prior to cancer vaccination protocols [102]. This property is not unique to cyclophosphamide but it is shared with other chemotherapeutics such as fludarabine and low dose weekly paclitaxel. High doses of chemotherapy do not have the same effect as they result in depletion of both Tregs and cytotoxic T cells. This strategy is sometimes referred to as "metronomic" chemotherapy and it's known to have anti-angiogenic effects as well. Clearly this represents a cheaper immunomodulating strategy, it is widely available worldwide, and it is used in several immunotherapy related clinical trials including in pancreatic cancer [58]. In addition, a strategy combining metronomic cyclophosphamide and an anti-CD25 monoclonal antibody has been successful in synergistically enhancing vaccine efficacy in a pancreatic adenocarcinoma mouse model [103].

CONCLUSION

The immune system plays a pivotal role in the progression of pancreatic cancer. While tumor immunology research in pancreatic cancer has traditionally lagged behind melanoma and renal cell carcinoma, this is largely due to a lack of focus rather than differences in the "immunogenicity" between the two types of cancer (although differences do exist). Several different immunotherapeutic strategies are now underway in various cancers and most, but not all strategies, are being tested in pancreatic cancer. It is likely that combination strategies will be necessary to both overcome the cancer induced immunosuppression (i.e., Treg depletion, anti-PD1, anti CTLA-4 antibodies) and target the cancer cells themselves (traditional chemotherapeutics, vaccination, ACT). Clinicians should be aware of both the mechanisms by which pancreatic cancer interacts with the immune system as well as the major treatment

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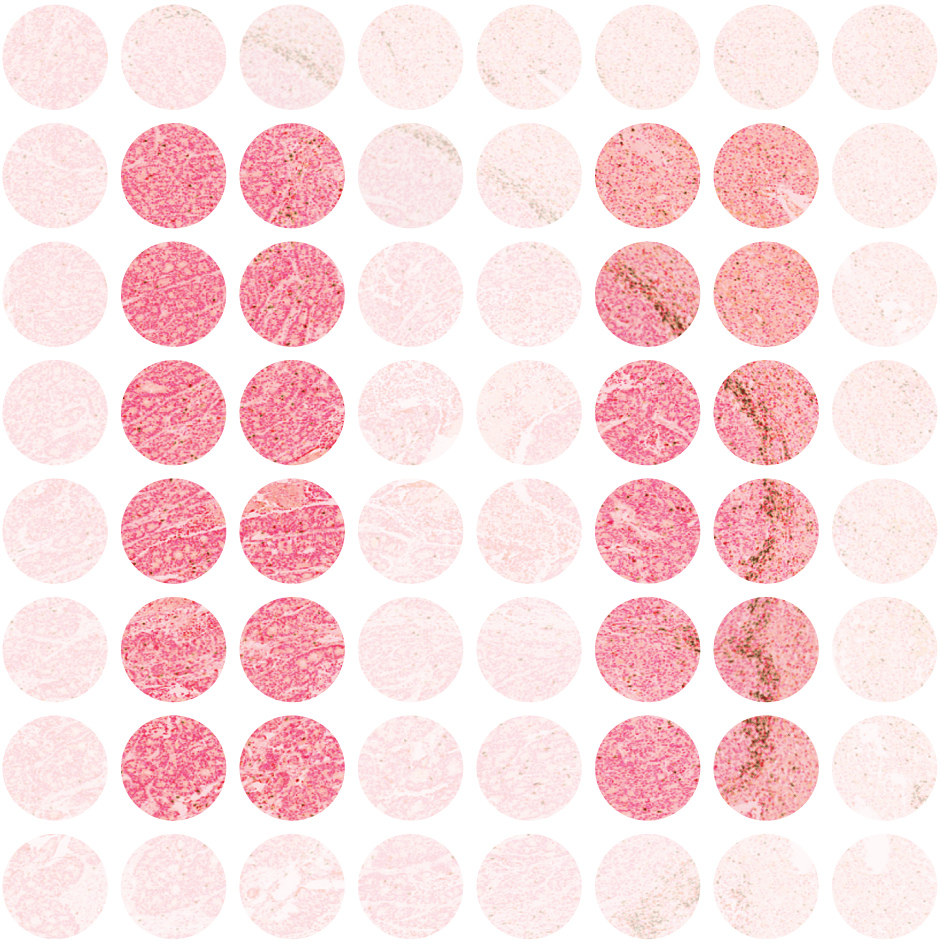
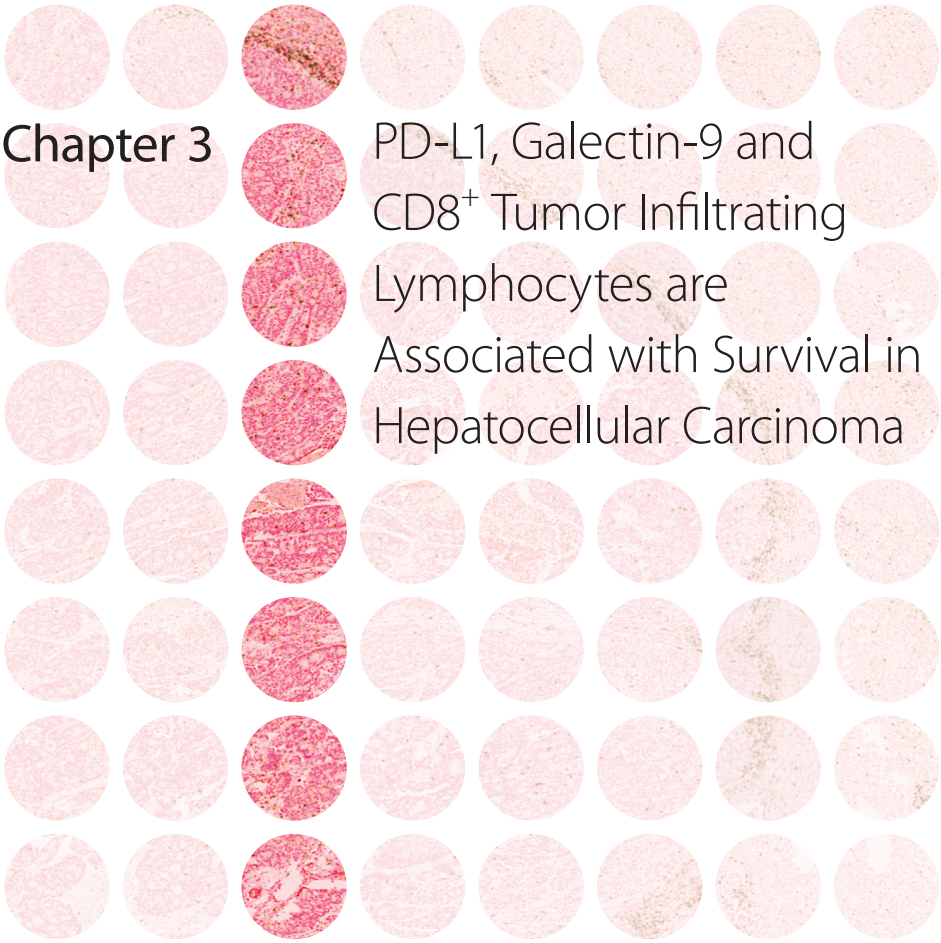


PART II

TUMOR TISSUE IMMUNE BIOMARKER EXPRESSION



Chapter 3

PD-L1, Galectin-9 and $CD8^+$ Tumor Infiltrating Lymphocytes are Associated with Survival in Hepatocellular Carcinoma

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ABSTRACT

Novel systemic treatments for Hepatocellular Carcinoma (HCC) are strongly needed. Immunotherapy is a promising strategy that can induce specific anti-tumor immune responses. Understanding the mechanisms of immune resistance by HCC is crucial for development of suitable immunotherapeutics. We used immunohistochemistry on tissue-microarrays to examine the co-expression of the immune inhibiting molecules PD-L1, Galectin-9, HVEM and IDO, as well as tumor $CD8^+$ lymphocyte infiltration in HCC, in two independent cohorts of patients. We found that at least some expression in tumor cells was seen in 97% of cases for HVEM, 83% for PD-L1, 79% for Gal-9, and 66% for IDO. In the discovery cohort (n=94) we found that lack of, or low, tumor expression of PD-L1 ($p<.001$), Galectin-9 ($p<.001$) and HVEM ($p<.001$), and low $CD8^+$TIL count ($p=.016$), were associated with poor HCC-specific survival. PD-L1, Galectin-9 and $CD8^+$TIL count were predictive of HCC-specific survival independent of baseline clinicopathologic characteristics and the combination of these markers was a powerful predictor of HCC-specific survival (HR 0.29; $p<.001$). These results were confirmed in the validation cohort (n=60). We show that low expression levels of PD-L1 and Gal-9 in combination with low $CD8^+$TIL count predicts extremely poor HCC-specific survival and it requires a change in two of these parameters to significantly improve prognosis. In conclusion, intra-tumoral expression of these immune inhibiting molecules was observed in the majority of HCC patients. Low expression of PD-L1 and Galectin-9 and low $CD8^+$TIL count are associated with poor HCC-specific survival. Combining immune biomarkers leads to superior predictors of HCC mortality.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death [1]. Curative treatments such as resection, local ablation or liver transplantation are only applicable in the 20% of patients with early stage disease [2]. For selected patients with advanced disease median survival can be modestly extended with the use of sorafenib [3]. However, cure at this stage is no longer possible.

Immunotherapeutic strategies, such as tumor vaccination, adoptive cell therapy and immune modulating antibodies, may provide alternative therapeutic options in HCC.[4,5] Indeed, immune modulating antibodies against CTLA-4 (Ipilimumab) or PD-1 (Nivolumab, Pembrolizumab) and PD-L1 (Atezolizumab) have already been approved for various cancers such as melanoma, lung, kidney and bladder cancer.[6-10] These so-called 'immune checkpoint' antibodies interrupt immune resistance mechanisms exploited by tumors to evade natural anti-tumor immunity. Reported immune resistance mechanisms include, among others, the expression of molecules that suppress intra-tumoral T-cell responses by ligating inhibitory receptors on T cells, such as PD-L1, galactin-9 and HVEM and the expression of enzymes that generate T-cell inhibitory metabolites, such as IDO.[11]

Binding of PD-L1 to its receptor PD-1, on activated T cells, suppresses T-cell responses.[12] PD-L1 is expressed in numerous tumors, including HCC, and *in-vitro* abrogation of the PD-1/PD-L1 interaction has been shown to reinvigorate tumor-specific responses of T-cells isolated from HCC patients.[13-19] Galectin-9 (Gal-9) is a carbohydrate-binding protein that is involved in T-cell homeostasis.[20] Contrasting effects of Gal-9 on anti-tumor immunity have been described. On the one hand binding of Gal-9 to its receptor TIM-3, expressed on activated T cells, causes T cell dysfunction and apoptosis in tumors[21] while binding of Gal-9 to CD44 promotes the differentiation of T-regulatory cells.[22] Conversely, Gal-9 can enhance T-helper 1 type anti-tumor immunity [23], inhibit NK cell chemotaxis to the tumor microenvironment[24] and exert anti-metastatic potential on tumor cells.[25,26] Like PD-L1, Gal-9 is expressed in several cancers, including HCC.[21,27] HVEM is a "molecular switch" with dual immune-stimulatory and inhibitory functions.[28] By ligation of LIGHT, HVEM stimulates T-cell responses, while binding to BTLA or CD160 leads to inhibition of T-cells. HVEM is known to be expressed in melanoma[29] and most recently in HCC.[30] IDO is the rate limiting enzyme in the catabolism of the essential amino-acid tryptophan.[31] Tryptophan depletion, as well as accumulation of tryptophan catabolites such as kynurenine, induce T-cell anergy and apoptosis. IDO inhibitors are currently in clinical development. IDO expression has been demonstrated in several cancer types, including HCC.[32]

As clinical efficacy of immune checkpoint antibodies seems dependent on expression of their target molecules in tumors[33,34] knowledge of their expression patterns in HCC may establish which of these molecules, or combinations, might be promising to target. No published study has systematically examined co-expression of multiple immune inhibitory molecules in a homogeneous cohort of HCC patients before. Thus, the primary aim of the present study was to examine the patterns of co-expression, as well as the relationship with the tumor infiltrating lymphocytes (TIL) and cancer-related survival, of several immune inhibitory molecules in HCC tumor tissue and adjacent non-tumorous tissue. PD-L1, Gal-9, HVEM and IDO were chosen for study because their mechanism of interaction with the immune system is generally understood while at the same time a reliable primary antibody is available. Tissue microarrays were constructed, and expression of the above molecules on tumor cells and hepatocytes was examined, by immunohistochemistry, in two separate patient cohorts.

PATIENTS AND METHODS

Patient population and tissue samples

Archived formalin fixed paraffin-embedded tissue samples from 154 patients who underwent hepatic resection for HCC at Erasmus MC-University Medical Center (EMC, n=94) or Amsterdam Medical Center (AMC, n=60), between June 2001 and July 2014, were used for this study. Fresh frozen tissue, which was available from 20 additional patients, was used for RNA extraction. Patients with HCC were selected for the study if they had undergone surgery with curative intent. Clinical information was collected retrospectively from the electronic record. The clinical information collected included etiologic factors, HCC recurrence, patient death, cause of death, as well as known prognostic clinicopathologic characteristics, such as tumor differentiation, vascular invasion, number of lesions, largest tumor size and pre-operative α-fetoprotein (AFP) level. The study was approved by the local medical ethical committee. In addition, the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Tissue microarray (TMA) construction

Three or four 0.6mm cores were taken from the tumorous area of 154 patients and two 0.6mm cores were taken from the corresponding tumor free liver (TFL) area of 133 of these patients. Areas with vital tumor and TFL tissue were marked by experienced pathologists (KB or JV) using archived H&E glass slides. The TMAs were made using either an automated tissue-arrayer ATA-27 (Beecher Instruments, Silver Springs MD, USA) or a fully manual tissue-arrayer MTA-1 (Beecher Instruments).

Immunohistochemistry and scoring

Complete information on the immunohistochemistry protocols can be found in the supplementary methods section. Complete information in the primary antibodies used can be found in Supplementary Table 1. Scoring was performed by two independent investigators (KS and HS) blinded to clinical outcome and differences resolved by mutual agreement. Scoring was performed on cancer cells (tumor cores) or hepatocytes (TFL cores). Intensity was scored in a scale from zero to three. Intra-core heterogeneity of staining intensity of tumor cells or hepatocytes was rarely observed in our cohort, thus only staining intensity was taken into consideration. In the case of CD8 staining the positive cells per core were counted manually and average counts were used for analysis.

Table 1. Patient characteristics

Characteristics	Discovery cohort N=94	Validation cohort N=60
Male/Female (%)	63/31 (67/33)	48/12 (80/20)
Hepatitis-B[a]/Hepatitis-C[b] (%)	23/11 (25/12)	14/19 (23/32*)
Cirrhosis (%)	32 (34)	20 (33)
Tumor differentiation (1-3)	26/47/20 (28/50/22)	15/34/10 (25/58/17)
Vascular invasion	58 (68*)	13 (42)
Single lesions (%)	72 (77)	50 (83)
Median size (Range)	5.9 cm (0.5-25.0)	5.0 cm (1.0-29.0)
Median AFP (Range)	8.5 ug/l (1-63.000)	9.0 ug/l (2-29.000)
Recurrence	50 (53)	28 (47)
Death	44 (47)	21 (35)
HCC related death	29 (31)	13 (22)

[a]HBsAg(+) and/or anti-HBc positive, [b]anti-HCV positive

*There is statistically significant more Hepatitis-C in the AMC cohort and vascular invasion in the EMC cohort.

PCR amplification

All primers for the target genes were intron spanning and sequences are listed in Supplementary Table 2. The RT-PCR amplification technique and primer sources are described in detail in the supplementary methods section.

Statistical analysis

All analysis was performed in duplicate. The discovery phase was performed in the EMC cohort while the validation phase was performed in the AMC cohort. The differences in expression of immune inhibitory molecules between tumor and TFL tissue was analyzed with the paired T-test. The associations between clinicopathologic parameters with the expression of immune inhibitory molecules, as well as the co-expression of the immune inhibiting molecules with each other, were examined using the $\chi 2$ tests or the T-test

Table 2. Cox-proportional Hazard regression analysis of patients' HCC-specific survival in the discovery cohort

	Univariate			Multivariate		
Variables	HR	95% CI	p-value	HR	95% CI	p-value
AFP>100 ug/l	2.67	1.17-6.13	**.020**	4.83	1.85-12.6	**.001**
One vs multiple lesions	3.53	1.63-7.67	**.001**	2.37	1.01-5.56	**.048**
PD-L1	0.19	0.86-0.43	**<.001**	0.30	0.13-0.72	**.007**
Gal-9	0.31	0.15-0.66	**.003**	0.33	0.14-0.80	**.014**
HVEM	0.21	0.09-0.50	**<.001**	0.50	0.20-1.25	.718
$CD8^+$TIL count	0.22	0.05-0.93	**.040**	0.18	0.04-0.82	**.027**

as appropriate. Survival (time to recurrence or HCC-specific death) was calculated from the date of surgery to the date of event (recurrence or death from HCC respectively), or the date of last follow up. Patients lost to follow-up were censored as of the last day of follow-up. Patients who died from causes other than HCC were censored at the date of death. Survival curves were estimated by the Kaplan-Meier method. For sensitivity analysis the survival analysis was repeated by excluding the patients who died from post-operative complications within 3 months after surgery. Optimal high vs low values were established by examining a grid of cutoffs and choosing the cutoff with the lowest -2 log likelihood, taking into consideration to maximize the proportion of patients identified by the cutoff value when possible. The Breslow test was used to asses differences between survival curves of different groups, while for parameters with three or four linearly associated levels the linear trend for factor levels was used. Given that we examined the expression of five individual parameters (PD-L1, Gal-9, HVEM, IDO, $CD8^+$TIL count) Bonferroni's correction required a p-value of < .01 for statistical significance in the discovery cohort. For multivariate analysis, the Cox proportional Hazard regression analysis was used. The statistical analysis was performed using the SPSS© 21 software.

RESULTS

Patient cohorts and baseline clinicopathologic characteristics

Clinicopathologic characteristics of patients in the EMC discovery cohort and the AMC validation cohort can be seen in Table 1, while complete information on etiology of liver disease can be found in Supplementary Table 3. Median time to recurrence and overall survival were 19.4 months and 37.2 months for the EMC cohort and 23.5 months and 31.8 months for the AMC cohort. Recurrence was seen in seventy-eight patients while forty-two patients died from HCC. A complete list with the causes of death can be found in Supplementary Table 4. From the known clinicopathologic prognostic factors an

α-fetoprotein (AFP) level above 400 μgl^{-1} (p=.002), multiple lesions (p=.043) and tumor size >3cm (p=.024) were associated with poor HCC-specific survival in the combined cohort (Supplementary Figure 1). Tumor grade and vascular invasion were not associated with HCC-specific survival.

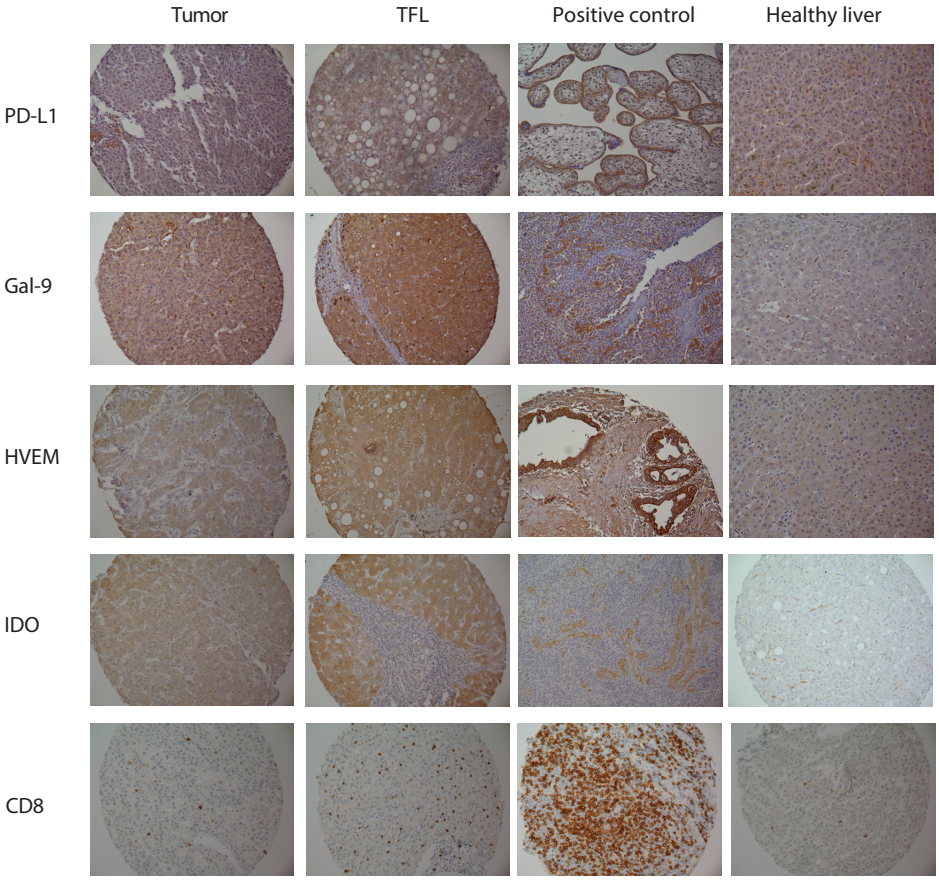

Figure 1. Representative stainings of tumor tissues and corresponding TFL tissues, positive control tissues, and normal liver. Positive controls tissues shown are placenta for PDL1, pancreatic cancer for HVEM, and tonsil for Gal-9, IDO and CD8. The positive control tissues stain as expected from prior literature. Note the hepatocyte staining seen for all molecules (except CD8) in the TFL tissue area and the general lack of hepatocyte staining in normal liver tissue. For Gal-9 characteristic Kupffer cell staining can be seen in the normal liver tissue.

Description of immune inhibitory molecule expression in the combined cohorts

Figure 1 shows representative stainings in tumor tissue, TFL tissue, positive control tissues and healthy liver tissues, while Supplementary Figure 2 shows examples of various expression levels observed in tumor tissues, for all the molecules examined in our study.

At least some cytoplasmatic expression in tumor cells was seen in 82.9% of cases for PD-L1, 78.8% for Gal-9, 96.6% for HVEM and 66.4% for IDO. In addition to their expression in the tumor tissues, PD-L1, Gal-9, HVEM and IDO were also expressed by hepatocytes in the surrounding TFL tissue. At least some cytoplasmatic expression in hepatocytes, in TFL tissue, was seen in 95.8% of cases for PD-L1, 93.5% for Gal-9, 100% for HVEM and 92.6% for IDO. No relationship was found between the expression of any molecule in tumor, or in the TFL tissue, with etiologic factors, or other clinicopathologic characteristics, after Bonferroni correction (Supplementary Table 5). Tumor expression correlated with TFL tissue expression in all cases. Figure 2 depicts the level of expression of each molecule in tumor cells and in hepatocytes in corresponding TFL tissues for the combined cohort. Note that there is a statistically significant under-expression of all molecules in the tumor tissue. In order to confirm that these molecules are indeed expressed in TFL tissues, we quantified mRNA expression in paired tumor and TFL tissues of 20 resected HCC patients, from which fresh frozen tissue was available. In Supplementary Figure 3 we show that mRNA encoding for all these immune molecules is indeed expressed in the TFL tissues, at levels comparable to the levels seen in the tumor tissues. Our results indicate that the investigated immune inhibitory molecules are frequently expressed not only in the tumor, but also in the TFL compartment of HCC patients.

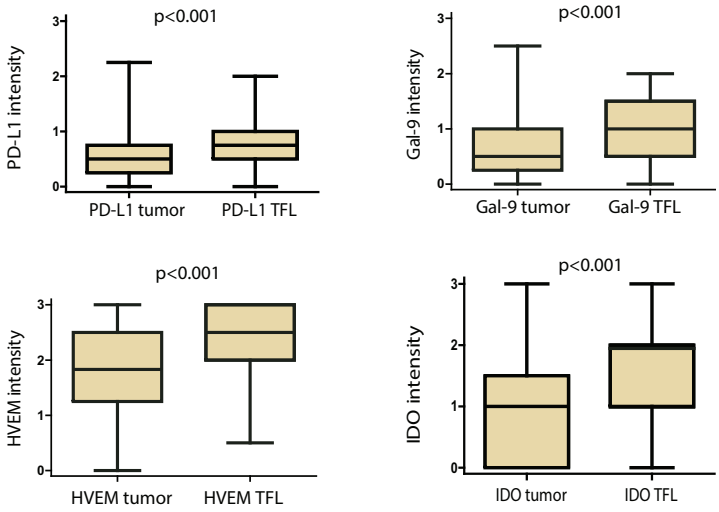

Figure 2. Comparison of protein expression levels of PD-L1, Gal-9, HVEM and IDO in the tumor tissues and the corresponding TFL tissues in the combined cohort. Boxplots representing protein expression in tumor cells vs TFL tissue. p values were determined by the paired T-test.

Immune inhibitory molecule expression: association with survival and recurrence in the discovery (EMC) cohort

Next we investigated the relationship between immune inhibitory molecule expression and survival in the EMC cohort. Optimal cut-offs for low versus high staining were made as described in the statistical methods. Patients were considered to have low PD-L1 (n=16) or Gal-9 (n=18) staining when there was complete absence of staining, while low HVEM included patients with either complete absence of staining or very faint staining (n=9). Low IDO (n=63) included patients with complete absence, or at most +1, staining intensity. We found that expression in tumor tissue, but not in surrounding TFL tissue, was significantly associated with HCC-specific survival for three out of the four molecules examined (Figure 3A). Specifically, no or low tumor expression of PD-L1 ($p< .001$), Gal-9 ($p< .001$) and HVEM ($p< .001$) was associated with poor HCC-specific survival, while expression of IDO was not associated with HCC-specific survival ($p= .953$). Hazard ratios and 95% confidence intervals can be found in Table 2. In addition, we quantified the numbers of $CD8^+$TIL. The relationship of $CD8^+$TIL count with HCC-specific survival was not significant after Bonferroni's correction ($p= .016$). For HCC recurrence the relationship with expression of these molecules followed the same trends (Supplementary Figure 4). Low tumor expression of PD-L1 ($p< .001$), Gal-9 ($p= .009$), HVEM ($p= .004$), and also low $CD8^+$TIL count ($p= .007$) were significantly associated with shorter time to HCC recurrence, while tumor expression of IDO was not associated with recurrence after Bonferroni's correction ($p= .029$). The results did not differ when analyses was performed by excluding the 9 patients who died at EMC in the post-operative period from causes other than HCC.

Based on a multivariate analysis, where all parameters with p values < .02 were entered in a single model, we derived that tumor expression of PD-L1 and Gal-9, $CD8^+$TIL count, AFP level and number of lesions were independent predictors of HCC-specific survival in the EMC cohort (Table 2). HVEM was not independently associated with HCC-specific survival when examined together with the other immune parameters.

Because of the ability of the immune inhibitory molecules to predict HCC-specific survival we wondered whether combining all three independently prognostic immune parameters would improve the prediction. Figure 4 shows that low levels of two or three of these parameters in tumor tissue predicts poor survival, while low level of none, or only one, parameter predicts good HCC-specific survival. Concordantly, the combination of PD-L1, Gal-9 and $CD8^+$TIL as a single biomarker was a powerful independent predictor of HCC-specific survival in multivariate analysis ($p< .001$, HR 0.29, 95%CI 0.18-0.48).

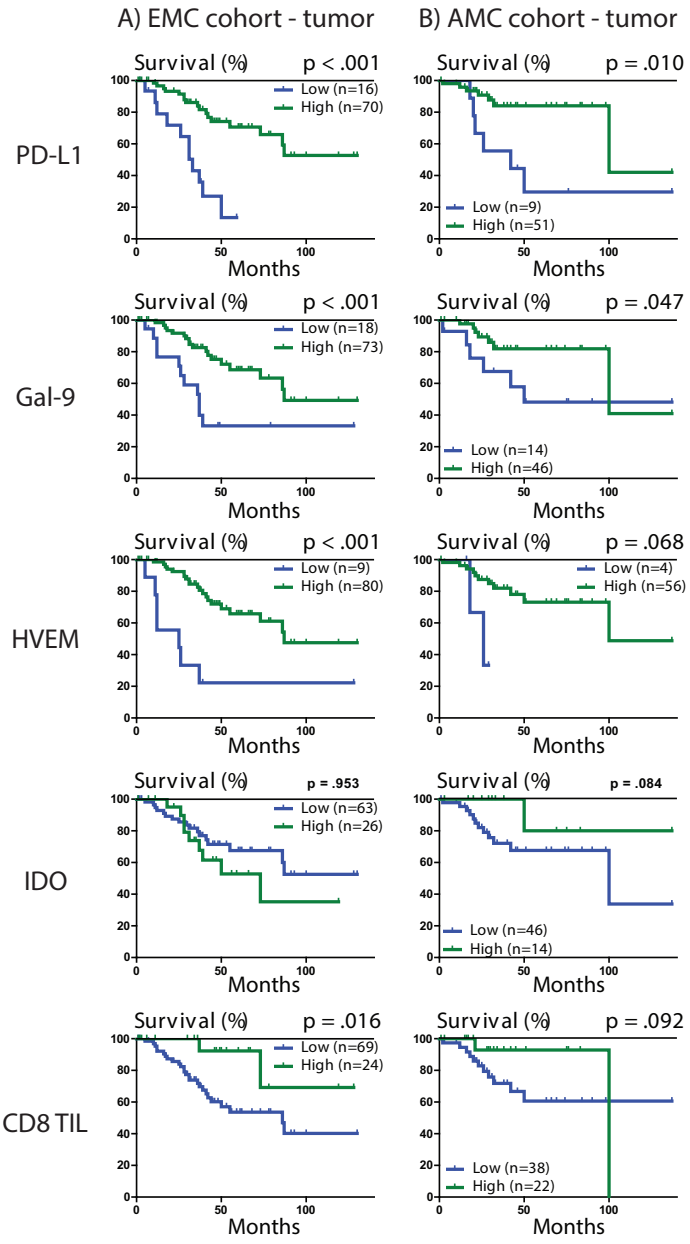

Figure 3. HCC-specific survival Kaplan-Meier curves of PD-L1, Gal-9, HVEM, IDO and CD8$^+$TIL count in the tumor and TFL compartments. (A) Survival curves in relation to tumor expression for the discovery (EMC) cohort. (B) Survival curves in relation to tumor expression for the validation (AMC) cohort. Optimal high vs low values were established by examining a grid of cutoffs and choosing the cutoff with the lowest -2 log likelihood. For determination of the p values Breslow test was used.

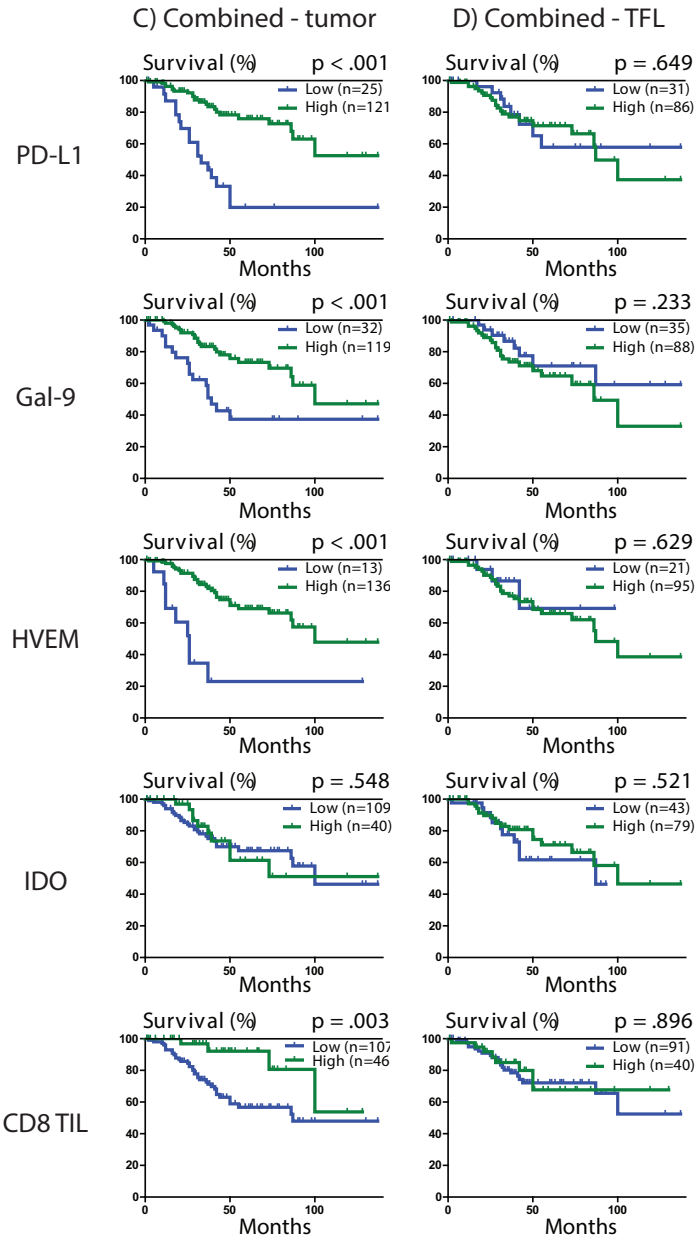

Figure 3 (continued). (C) Survival curves in relation to tumor expression for the combined cohorts. (D) Survival curves in relation to TFL tissue expression for the combined cohorts. Optimal high vs low values were established by examining a grid of cutoffs and choosing the cutoff with the lowest -2 log likelihood. For determination of the p values Breslow test was used.

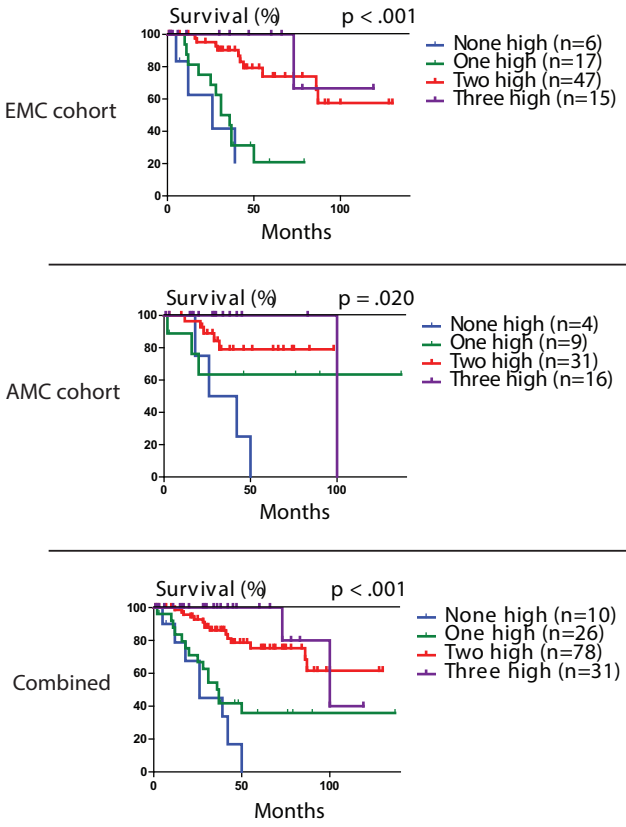

Figure 4. HCC-specific survival Kaplan-Meier curves of the combined PD-L1, Gal-9 and CD8$^+$TIL count biomarker. Combination of PD-L1, Gal-9 and CD8$^+$TIL count in relation to HCC-specific survival in the discovery (EMC) cohort, validation (AMC) cohort and combined cohort. For determination of the p values the linear trend for factor levels was used.

Validation in the AMC cohort

External validation of the above findings was performed in the cohort from AMC. Low tumor expression of PD-L1 (p= .010) and Gal-9 (p= .047) were also significantly associated with poor HCC-specific survival (Figure 3B). Low tumor CD8$^+$TIL count showed a trend towards poor HCC-specific survival (p= .092), with a hazard ratio (HR 0.36, 95%CI 0.08-1.64) that was of similar magnitude and direction as in the discovery (EMC) cohort (HR 0.22, 95%CI 0.05-0.93). The combination of PD-L1, Gal-9 and CD8$^+$TIL as a single biomarker (Figure 4) predicted HCC-specific survival (p= .020, HR 0.43, 95%CI 0.24-0.77) and was validated as an independent predictor of HCC-specific survival in multivariate analysis in the AMC cohort (p= .005).

Co-expression patterns and survival

Low levels of expression of either PD-L1, Gal-9 or HVEM were significantly associated with low levels of expression of the other 2 ligands (Supplementary Table 6). Such a relationship was not seen with IDO. The $CD8^+$TIL count showed a significant but weak correlation with PD-L1 expression (p= .046) but not with Gal-9, HVEM or IDO. Specifically, high expression of PD-L1 was correlated with a high $CD8^+$TIL count and vice-versa.

Having established the predictive power of the individual and combined expression of immune inhibitory molecules we wondered how the relationship of each molecule to survival related to tumor lymphocyte infiltration. It has been previously shown that tumor expression of PD-L1 in melanoma carries different prognostic values in the setting of high vs low TIL counts [33,35]. Figure 5A shows that low PD-L1 tumor expression in combination with low $CD8^+$TIL count is associated with very poor HCC-specific survival while high PD-L1 and high $CD8^+$TIL count is associated with good HCC-specific survival (p< .001, HR 0.29, 95%CI 0.17-0.50). The presence of either high PD-L1 or high $CD8^+$TIL count alone is associated with intermediate survival. A similar observation was made for the combination of Gal-9 tumor expression and $CD8^+$TIL count (p< .001, HR 0.29, 95%CI 0.17-0.49) (Figure 5B).

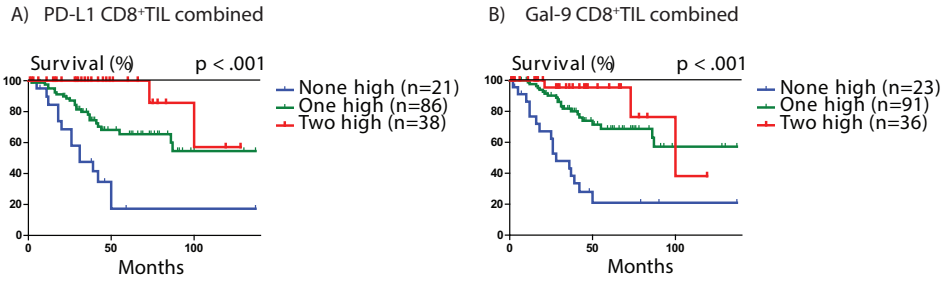

Figure 5. HCC-specific survival Kaplan-Meier curves of tumor PD-L1 and Gal-9 expression in relation to $CD8^+$TIL count in the combined cohorts. (A) Combination of PD-L1 and $CD8^+$TIL count in relation to HCC-specific survival. (B) Combination of Gal-9 and $CD8^+$TIL count in relation to HCC-specific survival. For determination of the p values the linear trend for factor levels was used.

DISCUSSION

In two independent cohorts, we found that low tumor expression of PD-L1 and Gal-9, as well as low $CD8^+$TIL count, are independent predictors of poor HCC-specific survival. HVEM expression, on the other hand, while individually an independent predictor of HCC-specific survival, was not independent when other immune parameters were taken into consideration. The combination of tumor PD-L1 and Gal-9 expression and $CD8^+$TIL

count appeared to be a powerful independent predictor of HCC-specific survival. We show that there is a group of patients with extremely poor prognosis who express low levels of PD-L1 and Gal-9 and have low $CD8^+$TIL count. It requires a change in two of these parameters to significantly affect prognosis.

Similar to our results, low expression of PD-L1 was found to be associated with poor survival in melanoma,[33] gastrointestinal-stromal tumors,[36] colorectal cancer,[37] and non-small cell lung cancer.[38] On the other hand, other studies in melanoma[39], colorectal[40] and renal cell cancer[41] have shown the opposite. Moreover, in a third melanoma study[42], and in studies in squamous-cell lung cancer,[43] urothelial cancer[44] and breast cancer,[45] tumor PD-L1 expression has shown no prognostic significance. In addition, and in contrast to our results, two prior studies in HCC have suggested that lower PD-L1 expression is associated with better survival,[15,18] while one recent study reported that lower PD-L1 staining is associated with better 2-year chance of recurrence.[46] These conflicting results on the association between PD-L1 expression and prognosis, are probably related to the lack of specificity of several anti-PD-L1 antibodies for immunohistochemical staining of PD-L1 in formalin-fixed paraffin-embedded tissues.[15,18,38,39,42] Specifically, in the study by Gao et.al.,[15] the PD-L1 clone MIH1 was used on paraffin-embedded HCC tissue. This particular clone has been shown to be non-specific on paraffin embedded tissue in prior studies [38,42]. To further confirm these prior observations we stained a number of control tissues and TMA cores with the MIH1 clone and compared the findings to our results. We show, in Supplementary Figure 5, that the MIH1 clone failed to properly stain placenta and tonsil tissue, and also provides positive stainings of tumor cores that were found to be negative for PD-L1 using the 9A11 antibody clone. Importantly, the anti-PD-L1 antibody that we used (clone 9A11) has been extensively validated for this purpose,[41,44,47,48] and we show that it provides the correct staining pattern in control tissues, namely selective staining of the syncytiotrophoblast layer in placenta tissue and staining of the crypt regions in tonsil tissue (Figures 1 and Supplementary Figure 5). Moreover, in a previous study,[49] we showed that this antibody gives similar, but more intense, staining patterns as the well-validated 5H1 clone.[50] The use of imaging software to analyze PD-L1 density, as performed in the studies by Gao et.al., and Wu et.al., is another possible explanation for the differing results.[15,18] Imaging software can inadvertently include expression of PD-L1 by stroma and TIL, in addition to tumor. Differences in the characteristics of the included patients, may be another factor. For example the study of Calderaro et.al.,[46] which examined a large cohort of patients using another validated anti-PD-L1 antibody, did not report on long term mortality endpoints, while information on recurrence was available in a little over half the patients, making direct comparisons with our study not possible. Finally, it is well known that both cytoplasmatic and membranous PD-L1 staining has been described in various cancers, and we are not the first to describe a

pre-dominance of cytoplasmatic staining over membranous staining for PD-L1. In fact, studies using well validated antibodies have demonstrated both types of staining in various cancers before[33,50] and other prior studies in HCC have also predominately demonstrated cytoplasmatic staining.[16] It has recently been hypothesized that cytoplasmatic tumor staining represents intracellular stores of PD-L1 ready to be transported to the membrane upon contact with immune cells.[33] Alternatively, cytoplasmic PD-L1 may be released as a functional soluble molecule into the extracellular microenvironment.[51]

Regarding the prognostic significance of Gal-9 expression in tumors there is more consensus. Similar to our findings, under-expression of Gal-9 has been associated with poor outcome in HCC,[27] and also in melanoma, breast, cervical and gastric cancers.[25,26,52,53] However, one recently published study in renal cell cancer[54] showed the opposite. It is possible that the significance of expression of Gal-9 may be tumor specific. In renal cell cancer for example the results for both PD-L1 and Gal-9 are opposite to ours.[41,54]

One may wonder why low expression of these molecules signifies high risk of HCC death, since their expression in itself is supposed to inhibit effector immune responses. It is known that several of these molecules are overexpressed in response to IFN-γ and TIL infiltration, a process called adaptive immune resistance.[33,55-57] Therefore, expression of immune inhibitory molecules in cancer cells may not only be induced by intrinsic mechanisms (i.e. mutations, epigenetics e.c.t.) but also by tumor-infiltrating immune cells. Probably, their presence in the tumor microenvironment reflects an active immunologic attack that is beneficial to patients. Low or no expression of such molecules could indicate that the cancer is beyond detection by the immune system or that the anti-tumor immune response is ineffective.

Several studies have shown that expression of PD-L1 is associated with TIL infiltration in various cancers.[33,38,43] Our study is the first to describe this association in HCC. Stratification of tumors, based on the expression of PD-L1 and the presence or absence of TILs, has been recently proposed.[33,35] We show that HCC patients with low tumor PD-L1 expression and $CD8^+$TIL infiltration (type II tumors), which suggests tumors with immune ignorance, according to the model by Teng et.al.,[35] have the worst prognosis (Figure 5A), while patients with high tumoral PD-L1 expression and TIL infiltration (type I tumors), which suggest adaptive immune resistance, have the best prognosis, as predicted. Taken together, our data demonstrate that HCC may behave according to this novel prediction model, and also suggest that type I tumors, that is tumors with an ongoing anti-tumor immune response (high PD-L1 and high TIL count), may be the ones to benefit from PD-L1 or PD-1 blockade. Indeed recent observations from clinical trials show that patients who express PD-L1 are the ones that seem to benefit from anti-PD1 therapy.[34] A similar

prediction model might be true for Gal-9 in HCC, as we show in Figure 5B. We support the idea that the use of TILs as a biomarker should be studied in the context of the expression of immune inhibitory molecules and that PD-L1 and Gal-9 are two of the molecules to be taken into consideration.

While the tumor expression status of these molecules in HCC has been reported, the expression of these molecules in the surrounding TFL tissue is much less understood. While PD-L1 and IDO have been shown, in single studies, to be indeed expressed in TFL tissue of patients with HCC,[13,58] that has never been shown for Gal-9 or HVEM before. We here show, both by immunohistochemistry and qPCR, that all four immune inhibiting molecules are present in both the tumor and the TFL compartments. We did not observe under-expression of mRNA levels in tumor tissue compared to TFL, while such an under-expression was seen by IHC. Possible explanations for the difference could be that we examined mRNA expression only in 20 paired samples. Another explanation could be translational and post-translational regulation of mRNA. The presence of these molecules in the TFL tissue of HCC patients may be explained by the production of cytokines by the infiltrating lymphocytes .[33,55-57] This observation suggests that therapies targeting these molecules may not only enhance anti-tumor immune responses but also anti-hepatitis-B virus or anti-hepatitis-C virus immune responses and thereby contribute to viral clearance. Conversely, such treatments harbor the theoretical danger of evoking undesired immunologic effects.

In our opinion, our study has several strong characteristics. We have studied a homogenous group of patients, have studied the expression of multiple immune inhibitory molecules in tumor, as well as surrounding TFL, tissue and have validated our findings in an independent cohort. Our study is the first to study the co-expression of multiple immune inhibitory molecules in HCC. There are of course also limitations in our study. Our selected panel of immune inhibitory molecules is by no means exhaustive. We focused on molecules for which there is solid evidence for their immune inhibitory role in cancer, for which a reliable antibody was available for immunohistochemistry and of which the corresponding mechanism of interaction with immune cells is well understood. However, as our understanding of the immune inhibiting mechanisms of cancer expands other molecules should be added. In addition, while the use of TMAs to study such questions has clear benefits, such as the rapid analysis of large number of tissue cores over identical experimental conditions and preservation of valuable patient tissue for future studies, there are also drawbacks. One is the inability of TMAs to represent the complex spatial interactions of immune inhibiting molecules in the complete tumor microenvironment. For this reason we did not evaluate the expression of immune inhibitory molecules by tumor infiltrating lymphocytes, as performed, for PD-L1, in the study

by Calderaro et.al.,[46] but focused on the expression of these molecules in tumor cells. Depending on the types of immune cells under study the relationship of expression of these molecules to outcome may be affected.[59] Another limitation of TMAs is that it is not ideal to evaluate the presence of uncommon cell types. While $CD8^+$ lymphocytes are abundant, $FoxP3^+$ T-regulatory cells are much less common and thus more difficult to systematically evaluate using TMAs.

In conclusion, we show that low tumor expression of PD-L1 and Galectin-9, as well as low $CD8^+$TIL count, are associated with poor HCC-specific survival in patients with resected HCC. PD-L1 and Galectin-9 expression in tumors may be induced in response to immunologic pressure which may explain why their presence is associated with prolonged survival. PD-L1 and Galectin-9 may be promising immunotherapeutic targets in HCC patients with tumors expressing these co-inhibitory molecules.

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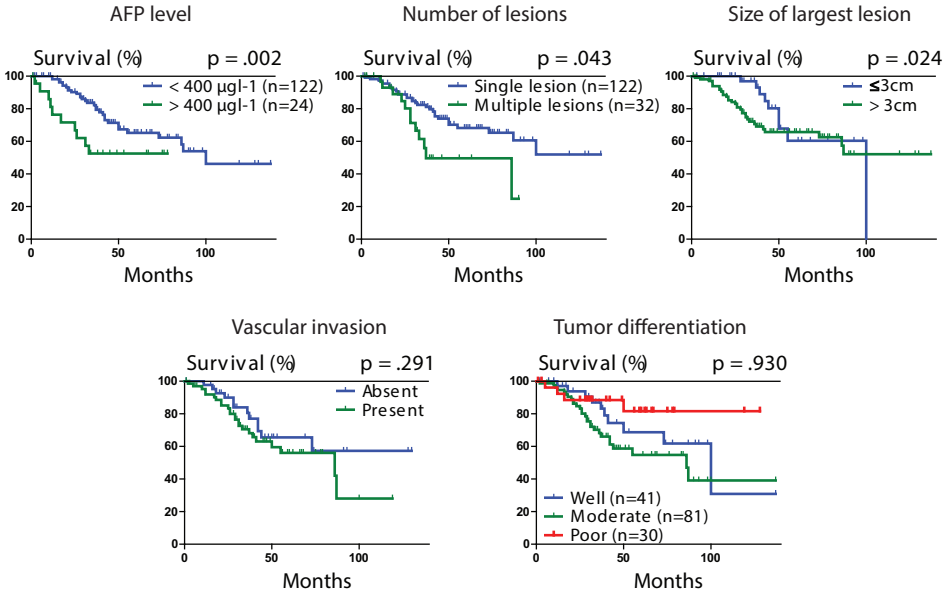
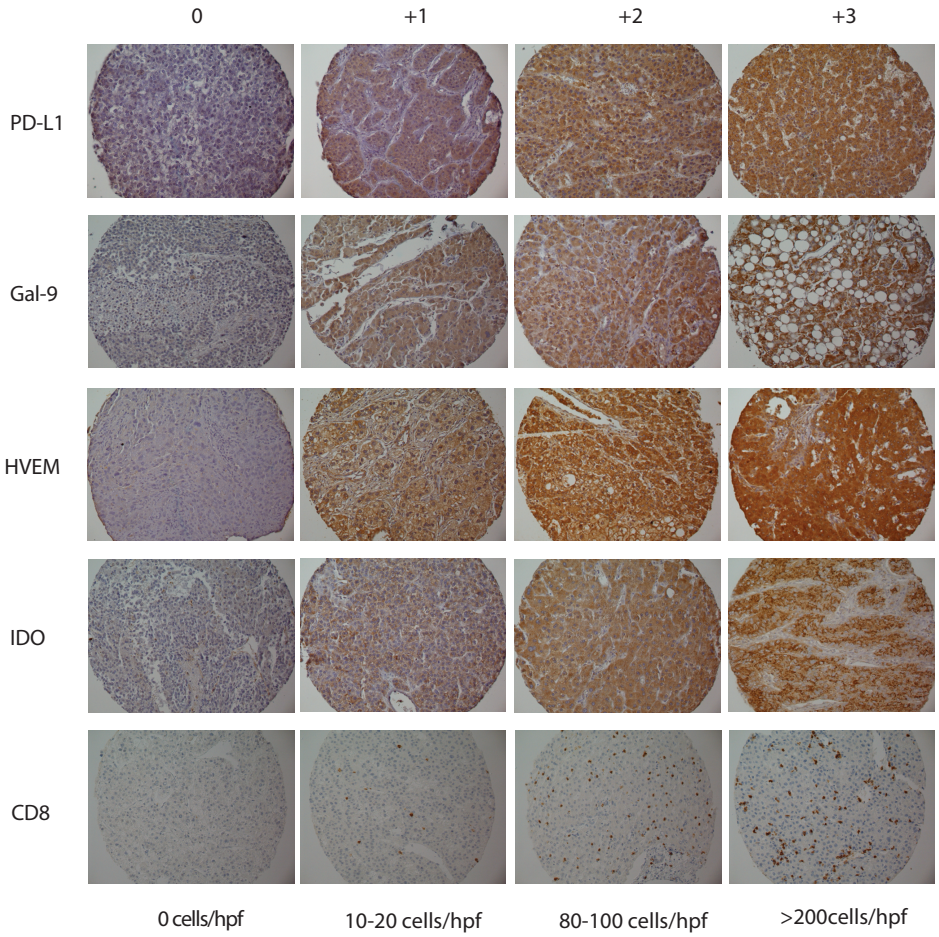
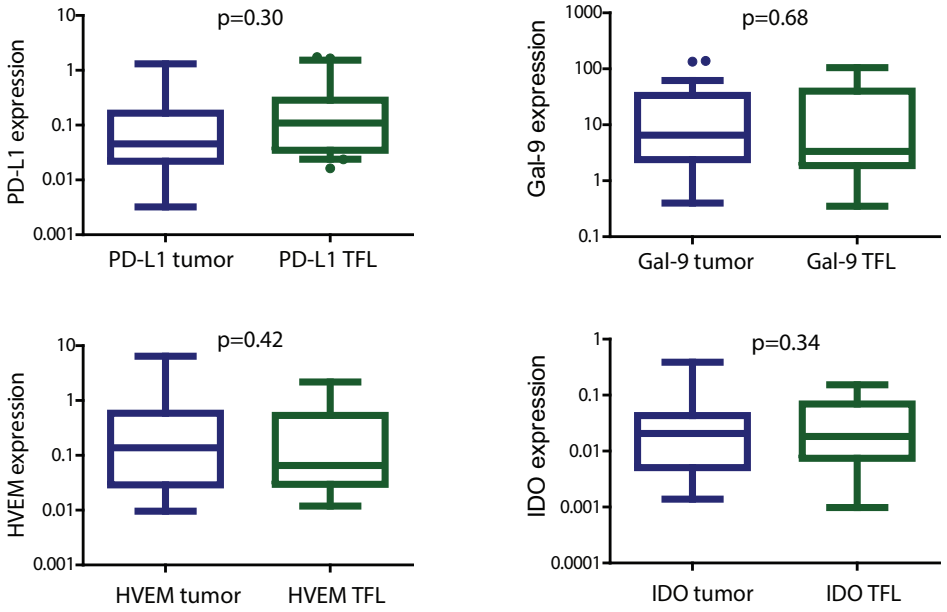
Supplementary Figures



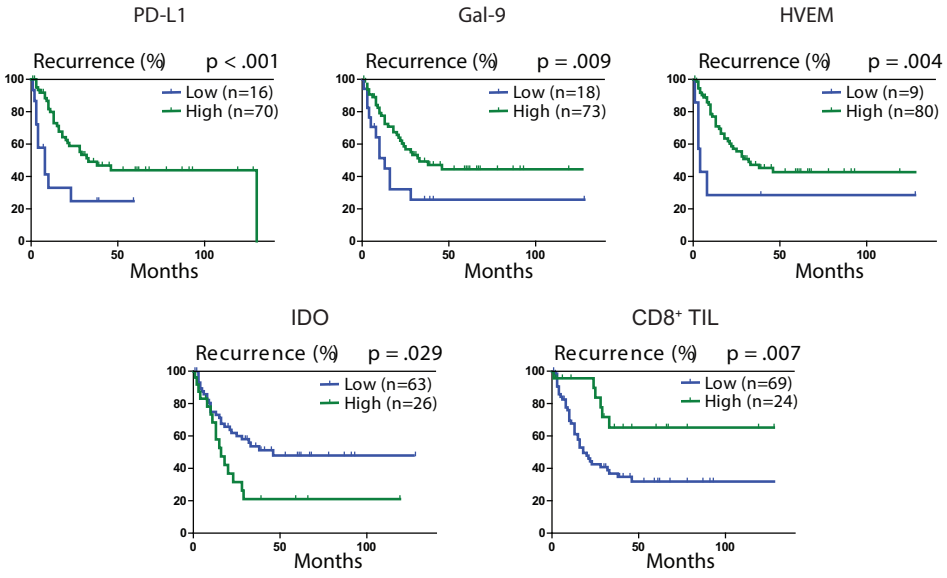
Supplementary Figure 1. Kaplan-Meier curves of HCC-specific survival in relation to baseline clinicopathologic characteristics for the combined cohort of patients.



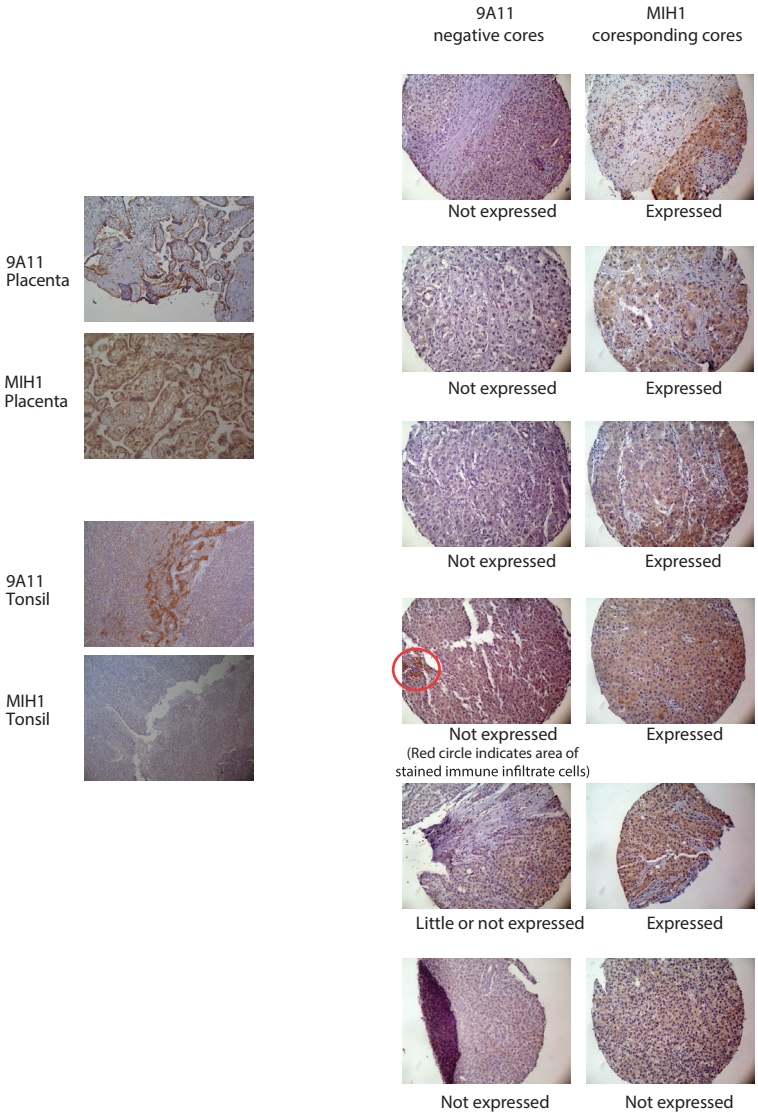
Supplementary Figure 2. Representative immunohistochemical stainings, showing variable expression of immune inhibitory molecules and CD8+TIL infiltration, in tumor tissues. Numbers in the top indicate intensity scores while numbers in the bottom indicate CD8+ counts per tissue core.



Supplementary Figure 3. mRNA expression of PD-L1, Gal-9, HVEM and IDO in tumor and TFL tissue. Boxplot of mRNA expression levels in 20 HCC patients with available fresh frozen tissue from the tumor and TFL area. Real Time RT-PCR data are corrected with the geomean of three housekeeping genes: GUS, PMM1, HPRT1. Note that these 20 patients are not part of the study cohort.



Supplementary Figure 4. Kaplan-Meier curves of time to recurrence in relation to immune inhibitory molecule expression in tumors for the EMC cohort.



Supplementary Figure 5. Comparison between the anti-PD-L1 antibody clones 9A11 and MIH1. Note that the MIH1 antibody does not selectively stain trophoblastic cells in human placenta FFPE tissue or the crypt regions of human tonsil FFPE tissue. In addition, in 5/6 cores, HCC tumor cells not stained with the 9A11 clone are stained with the MIH1 clone. This indicates a lack of specificity for the MIH1 clone.

Supplementary tables

Supplementary table 1. Primary antibodies used

Antigens	Antibody source	Clone	Retrieval buffer	Dilution
PD-L1	Dr. G. Freeman[a]	405.9A11	Tris EDTA	1:50
Gal-9	R&D systems[b]	Goat polyclonal	Tris EDTA	1:200
HVEM	Millipore[c]	2G6-2C7	Citric acid	1:200
IDO	Millipore[d]	10.1	Citric acid	1:200
CD-8[e]	Dako[f]	C8/144b		

[a] Kindly provided by Dr. Gordon J Freeman, Dana-Farber Cancer Institute, Boston, MA *Choueiri TK et.al., Ann Oncol.2014: 25:2178-84*

[b] http://www.rndsystems.com/Products/AF2045 *Mengshol JA et. al.PLoS One.2010: 5:e9504*

[c] http://www.merckmillipore.com/NL/en/product/Anti-TNFRSF14-Mouse-mAb-%282G6-2C7%29,EMD_BIO-AP1159?CategoryName=000000260002b67900020023&CategoryDomainName=Merck-MerckMillipore

[d] http://www.merckmillipore.com/NL/en/product/Anti-Indoleamine-2%2C3-dioxygenase-Antibody%2C-clone-10.1,MM_NF-MAB5412?bd=1#documentation *Soliman H et.al., Cancer Immunol Immunother. 2013: 62: 829–837*

[e] CD8 staining performed under standard clinical laboratory conditions

[f] http://www.dako.com/nl/ar38/p102650/prod_products.htm?setCountry=true&purl=ar38/p102650/prod_products.htm?undefined&submit=Accept%20country *Mason DY, et.al., J Clin Pathol 1992:45:1084-8*

Supplementary Table 2. Primers used

Gene	Symbol	Forward primer	Reverse primer
Glucuronidase, beta	GUSB	5′-CAGGTGATGGAAGAAGTGG-3′	5′-GTTGCTCACAAAGGTCACAG-3′
Phosphomannomutase 1	PMM1	5′-CGAGTTCTCCGAACTGGAC-3′	5′-CTGTTTTCAGGGCTTCCAC-3′
Hypoxanthine phosphoribosyltransferase 1	HPRT1	5′-GCTATAAATTCTTTGCTGACCTGCTG-3′	5′-AATTACTTTTATGTCCCCTGTTGACTGG-3′
TNF receptor superfamily, member 14	HVEM	5′-CACCGAGAGTCAGGACAC-3′	5′-GAAACCACCATACCCAGTG-3′
Lectin, galactoside-binding, soluble, 9	Gal-9	5′-TCTGGGACTATTCAAGGAGGTC-3′	5′-CCATCTTCAAACCGAGGGTTG-3′
CD274	PD-L1	5′-GTGACCAGCACACTGAGAAT-3′	5′-CCAGAATTACCAAGTGAGTCC-3′
Indoleamine 2,3-dioxygenase 1	IDO1	5′-GCCAGCTTCGAGAAAGAGTTG-3′	5′-ATCCCAGAACTAGACGTGCAA-3′

Supplementary Table 3. Etiology of liver disease*

Type of liver disease	N (%)
No known liver disease	50 (32.5)
Hepatitis B	30 (19.5)
Hepatitis C	30 (19.5)
Alcoholic liver cirrhosis	15 (9.7)
NASH	14 (9.1)
Hemochromatosis	5 (3.2)
Cryptogenic cirrhosis	3 (1.9)
Primary biliary cirrhosis	2 (1.3)
Porphyria	1 (0.6)

*When two etiologic factors where present in a single patient only the most dominant etiologic factor was considered, as determined by an experienced hepatologist. Thus the liver disease of seven patients with Hepatitis-B sero-positivity was attributed to other concurrent etiologic factors (Hepatitis-C x4, alcoholic liver cirrhosis x2, NASH x1). Etiologic information was missing from 4 patients.

Supplementary Table 4. Causes of death

Cause of death	N (%)
HCC	42 (27.3)
Postoperative complications (<3 months post HCC surgery)	14 (9.1)
Other cancer (not HCC)[1]	4 (2.6)
Other cause (not HCC)[2]	5 (3.2)
Alive	89 (57.8)

[1]Lung cancer x1, urothelial carcinoma x1, cholangiocarcinoma x1, colorectal cancer x1
[2]Severe lung disease x1, cardiovascular x2, pneumonia x1, unknown (sudden death) but not HCC (negative workup just before death) x1

Supplementary Table 5. Correlations of immune biomarker expression with clinicopathologic characteristics

Clinicopathologic characteristics	PD-L1 Tumor	Gal-9 Tumor	HVEM Tumor	IDO Tumor	PD-L1 TFL	Gal-9 TFL	HVEM TFL	IDO TFL
Hepatitis-B	.247	.259	.540	.364	.459	.112	.210	.573
Hepatitis-C	.513	.334	.536	.411	.369	.288	.422	.056
Cirrhosis	.113	.571	.135	.045	.465	.032	.401	.024
Tumor differentiation	.429	.804	.768	.247	.963	.802	.541	.906
Vascular invasion	.122	.286	.408	.349	.008	.157	.174	.337
One vs multiple lesions	.446	.316	.045	.249	.246	.118	.509	.498
Tumor Size > 3cm	.315	.318	.037	.442	.413	.032	.109	.578
AFP>100 ug/l	.159	.157	.064	.065	.403	.297	.503	.105

Results provided as p-values. Note that after Bonferroni correction none of the associations reach statistical significance.

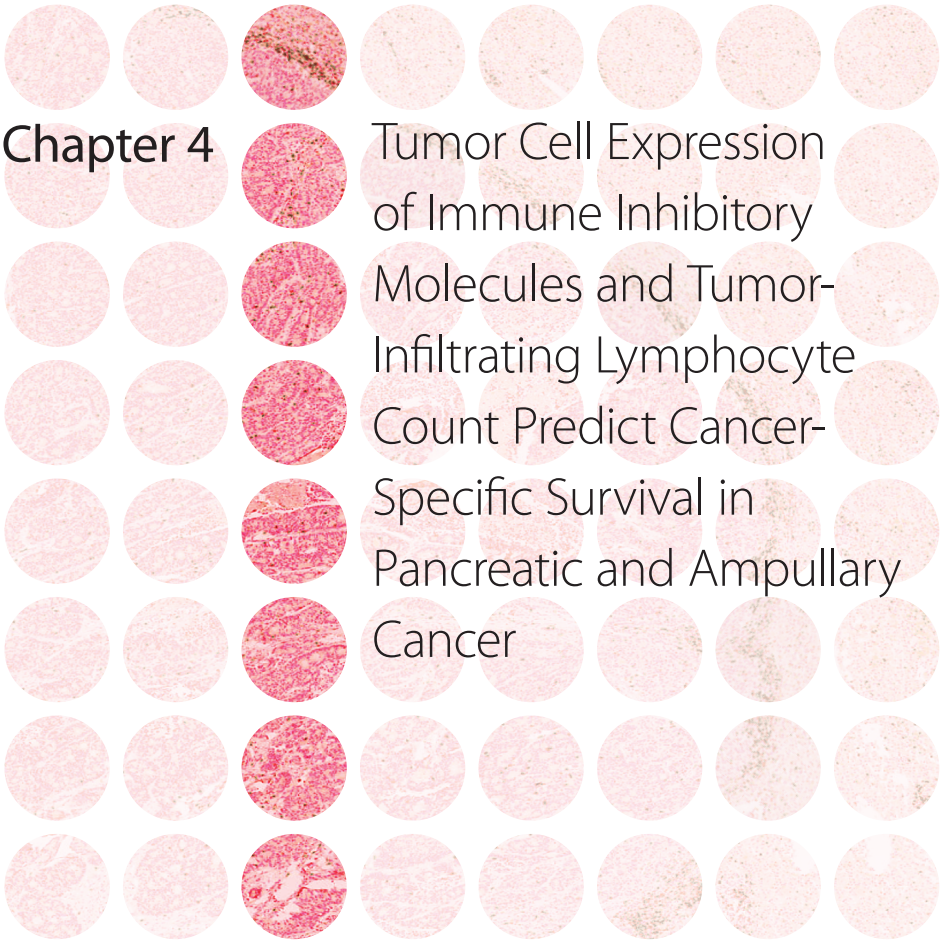
Supplementary table 6. Co-expression patterns of immune inhibitory molecules and $CD8^+$ TIL counts in tumors. Tables 6a, 6b, 6c are 2x2 tables showing co-expression patterns of inhibitory molecules . Table 6d is a 2x2 table of $CD8^+$ TIL count and PD-L1 expression

a		Gal-9		p =.001
		Low	High	Total
PDL1	Low	12	13	25
	High	20	101	121
	Total	32	114	146

b		HVEM		p <.001
		Low	High	Total
PDL1	Low	8	16	24
	High	5	116	121
	Total	13	132	145

c		HVEM		p <.001
		Low	High	Total
Gal-9	Low	10	22	32
	High	3	114	117
	Total	13	136	149

d		PD-L1		p =.046
		Low	High	Total
CD8	Low	21	83	104
	High	3	38	41
	Total	24	121	145



Chapter 4

Tumor Cell Expression of Immune Inhibitory Molecules and Tumor-Infiltrating Lymphocyte Count Predict Cancer-Specific Survival in Pancreatic and Ampullary Cancer

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Under submission

ABSTRACT

Understanding the mechanisms of immune resistance in pancreatic and ampullary cancers is crucial for the development of suitable biomarkers and effective immunotherapeutics. Our aim was to examine the expression of the immune inhibiting molecules PD-L1, Galectin-9, HVEM, IDO and HLA-G, as well as CD8+ and FoxP3+ tumor infiltrating lymphocytes (TIL), in pancreatic and ampullary cancers, and to relate their individual, as well as their combined expression, to cancer survival. Tumor tissue from 224 pancreatic and ampullary cancer resection patients was used to construct tissue-microarrays. Expression of immune inhibitory molecules and TIL was examined by immunohistochemistry. We show that immune inhibitory molecules are prevalently expressed. Moreover, high tumor expression of PD-L1 (p=.002), Gal-9 (p=.003), HVEM (p=.001), IDO (p=.049), HLA-G (p=.004) and high CD8/FoxP3 TIL ratio (p=.006) were associated with improved cancer-specific survival. All immune biomarkers, with the exception of IDO, were individually predictive of cancer-specific survival when adjusted for clinicopathologic characteristics. For every additional immune biomarker present survival was almost two-fold prolonged (HR 0.57 95%CI 0.47-0.69, p<.0001). We conclude that pancreas and ampullary cancers are rich in expression of immune-inhibitory molecules. These molecules can be targets for future immunotherapeutics, as well as form powerful immunological biomarkers. We propose that such immune biomarker panels be included in future prospective immunotherapy trials.

INTRODUCTION

Pancreatic cancer is a leading cause of cancer related death and more than 260,000 people die of the disease, every year, worldwide [1]. Due to its aggressive nature and late presentation 5-year survival is a dismal 6%. Only 15-20% of patients are candidates for surgical resection with the 5-year survival, in these patients, improving to 22%. Traditional chemotherapy is minimally effective, despite some recent success [2, 3]. Thus, novel therapeutic strategies against pancreatic cancer are needed.

The recognition of the critical importance of the immune system in cancer surveillance and elimination [4] has led to the development of various immunotherapeutic strategies against cancer [5]. Immune checkpoint inhibitors, such as antibodies against CTLA-4 or PD-1, have been the most successful to date [6]. These so-called immune checkpoint inhibitors interrupt immune resistance mechanisms exploited by tumors to evade natural anti-tumor immunity. Reported immune resistance mechanisms include, among others, the expression of molecules that suppress intra-tumoral T-cell responses, by ligating inhibitory receptors on T cells, such as PD-L1, galectin-9, HVEM or HLA-G, and the expression of enzymes that generate T-cell inhibitory metabolites, such as IDO. The tumor microenvironment of pancreatic cancer is rich in such immune suppressive mechanisms [7].

PD-L1 binds to its receptor PD-1 on activated T cells and suppresses T-cell responses [8]. PD-L1 is expressed in numerous tumors and in-vitro abrogation of the PD-1/PD-L1 interaction can reinvigorate tumor-specific T-cell responses. Galectin-9 (Gal-9) is involved in T-cell homeostasis [9] by either binding to TIM-3 expressed on activated T cells and causing T cell dysfunction and apoptosis [10] or by binding to CD44 promoting T-regulatory cell differentiation [11]. Conversely, Gal-9 can also enhance T-helper 1 type anti-tumor immunity [12] and exert anti-metastatic potential on tumor cells [13, 14]. HVEM is a "molecular switch" with dual stimulatory and inhibitory functions, although its inhibitory function is likely dominant [15]. Binding of HVEM to BTLA or CD160, members of the immunoglobulin (Ig) superfamily, leads to inhibition of T-cells [16]. IDO is the rate limiting enzyme in the catabolism of the essential amino-acid tryptophan. Tryptophan depletion, as well as accumulation of tryptophan catabolites such as kynurenine, induce T-cell anergy and apoptosis, while IDO inhibition enhances proliferation of TIL and decreases T-regulatory cells [17]. IDO inhibitors are currently in clinical development. Finally, HLA-G is a non-classical MHC class I molecule with a role primarily in preventing fetal rejection in pregnancy [18]. HLA-G and is known to induce, upon ligation of inhibitory receptors such as ILT-2 and ILT-4, inhibition of NK cells, $CD4^+$ and $CD8^+$ lymphocytes [19].

As clinical efficacy of immune checkpoint antibodies seems partially dependent on expression of their target molecules in tumors [20, 21] immunological biomarkers will likely become extremely useful as prognostic and/or predictive tools in the future. However, given the complexity of cancer, and that of the immune system, it is unlikely that a *single* biomarker, like PD-L1 for example, will be able to discriminate prognostically, or predict success to therapy, in a clinically useful manner. In fact, the role of PD-L1 as a single biomarker predicting efficacy of anti-PD-1 and anti-PD-L1 therapies has come into question. Thus the primary aim of the present study was to examine the expression and co-expression of multiple immune inhibitory molecules in pancreatic and ampullary cancer and relate their expression to the tumor infiltrating lymphocytes, baseline known adverse clinicopathologic factors and outcome. We hypothesized that the expression of combinations of these immune inhibitory molecules would create a strong immune biomarker panel. The molecules chosen for study (PD-L1, Gal-9, HVEM, IDO, HLA-G) were chosen because their mechanism of interaction with the immune system is generally known and because a reliable primary antibody is available.

MATERIALS AND METHODS

Patient population and tissue samples.

Archived formalin fixed paraffin-embedded tissue from 224 patients who underwent pancreatic cancer (n=148) or ampullary cancer (n=76) resection at the Erasmus University Medical Center Cancer Institute between December of 2000 and December 2014 were retrieved from the pathological archive. Supplementary Table 1 lists the exact histological diagnosis and precise anatomic location for each cancer. Baseline clinicopathologic characteristics and information on the use of chemotherapy, cancer recurrence and patient survival was retrospectively collected from the electronic record. The rational for including both patients with pancreatic and ampullary cancer in our study was that cancers arising from the ampulla of Vater are frequently clinically indistinguishable from, and often treated similarly with, cancers arising from the pancreas. Patients with duodenal and extrahepatic bile duct carcinomas, as well as all endocrine neoplasms were excluded. In addition we excluded patients who died from post-operative complications. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Medical Ethical Committee of Erasmus MC.

TMA construction.

Five 1-mm cores were taken from the tumorous area of 224 patients. The tumorous areas were marked by an experienced pathologist (KB) using archived H&E glass slides. The TMAs were made using an automated tissue-arrayer ATA-27 (Beecher Instruments, Silver

Table 1.

Baseline characteristics		N=224 (% or range)
Age		67.2 (33.4-85.2)
Gender (male/female)		132 (58.9) / 92 (41.1)
Positive margins [a]		81 (36.8)
Lymph node metastasis		139 (62.1)
CA-19.9 (kU/l)		67 (1-6,500)
Differentiation	Good	19 (8.5)
	Moderate	135 (60.5)
	Poor	69 (30.9)
Peri-neural invasion		136 (73.1)
Origin (pancreas/ampulla)		148 (66.1) / 76 (33.9)
T-stage [b] (pancreas/ampulla)	T1	8 (5.4) / 10 (13.2)
	T2	24 (16.2) / 19 (25.0)
	T3	116 (78.4) / 44 (57.9)
	T4	NA / 3 (3.9)
Adjuvant chemotherapy		82 (37.6)
Systemic chemotherapy [c]		99 (47.4)
Recurrence		146 (65.8)
Cancer specific death [d]		136 (60.7)

[a] Margins ≤1mm included as positive.

[b] T-stage classification differs between pancreas and ampullary cancers (AJCC/UICC 2010)

[c] Any systemic adjuvant or palliative chemotherapy

[d] 6 patients died from causes other than pancreas or ampulary cancer (cerebrovascular accident x2, myocardial infarction x1, multiple comorbidities x1, adjuvant chemo-radiation related x1, urothelial carcinoma x1).

Springs MD, USA). In order to ensure that the stainings observed in the TMA cores were representative of the entire tumor, ten tissue blocks were chosen from which full slides were cut and stained. The purpose was to compare the findings between the TMA cores and the full slides and assess if staining heterogeneity was an issue.

Immunohistochemistry.

Immunohistochemistry was performed as previously described [22, 23] (see also supplementary methods section). Complete information on the primary antibodies used can be found in Supplementary Table 2. For PD-L1 staining we used a validated anti-PD-L1 antibody (clone 9A11), and a subset of cores was also stained with a second validated anti-PD-L1 antibody (clone 5H1). Stainings of appropriate control tissues to evaluate the specificity of all antibodies can be found in Supplementary Figure 1.

Table 2. Univariate Cox proportional Hazard regression analysis of patients' survival

Variables*	HR	95% CI	p-value
Tumor differentiation	1.79	1.32-2.43	**<.001**
T stage	1.57	1.19-2.09	**.002**
Margin status	2.12	1.56-3.13	**<.001**
Lymph node status	3.24	2.16-4.85	**<.001**
CA-19.9 (log10)	1.49	1.18-1.95	**.001**
Peri-neural invasion	2.46	1.55-3.89	**<.001**
Pancreas vs ampulla	1.54	1.06-2.22	**.021**
Systemic chemotherapy	1.36	0.96-1.92	.086
Age	1.00	0.98-1.03	.910
IDO	0.58	0.35-1.00	**.049**
PD-L1	0.54	0.36-0.79	**.002**
Gal-9	0.58	0.41-0.83	**.003**
HVEM	0.45	0.28-0.72	**.001**
HLA-G	0.43	0.24-0.76	**.004**
$CD8^+$TIL	0.65	0.44-0.95	**.026**
$FoxP3^+$TIL	0.69	0.49-0.97	**.034**
CD8/FoxP3 ratio	0.55	0.36-0.84	**.006**

*In all cases hazard ratios reflect the presence, or higher level, of a clinicopathologic characteristic or high level of immune biomarkers. Note that the presence, or higher levels, of all clinicopathologic characteristics predicts worse survival (except for use of chemotherapy and age), while high levels of any of the immune biomarkers is protective.

Evaluation of immunohistochemistry staining.

Scoring focused on expression on cancer cells while expression on dysplastic, atypical or normal cells or TIL was noted but not scored. Figure 1 shows typical staining patterns, and respected scores, given for all molecules under study. Scoring was performed by two independent investigators (KS and KY) blinded to clinical outcome, and differences resolved by mutual agreement. Staining intensity was scored, for each core, from a scale of 0-3 and the proportion of positive cells was determined, for each core, in quadrilles. A composite score was given from a scale from 0-3 which reflected the cytoplasmatic staining intensity (scale 0-3) multiplied by the proportion of positive cells (0-100%) for the cases where focal expression was seen. For example if the staining intensity was +2 but the staining was only present in 50% of tumor cells then the composite score was 1. When membranous staining was detected this was separately scored using the same methodology. Average scores from the 5 tumor cores were used for analysis. For CD8 and FoxP3 the positive cells per core were counted manually and average counts were used for analysis. Cutoffs were optimized to produce the most discriminating biomarkers for cancer-specific survival, as further discussed in the statistical methods. For PD-L1

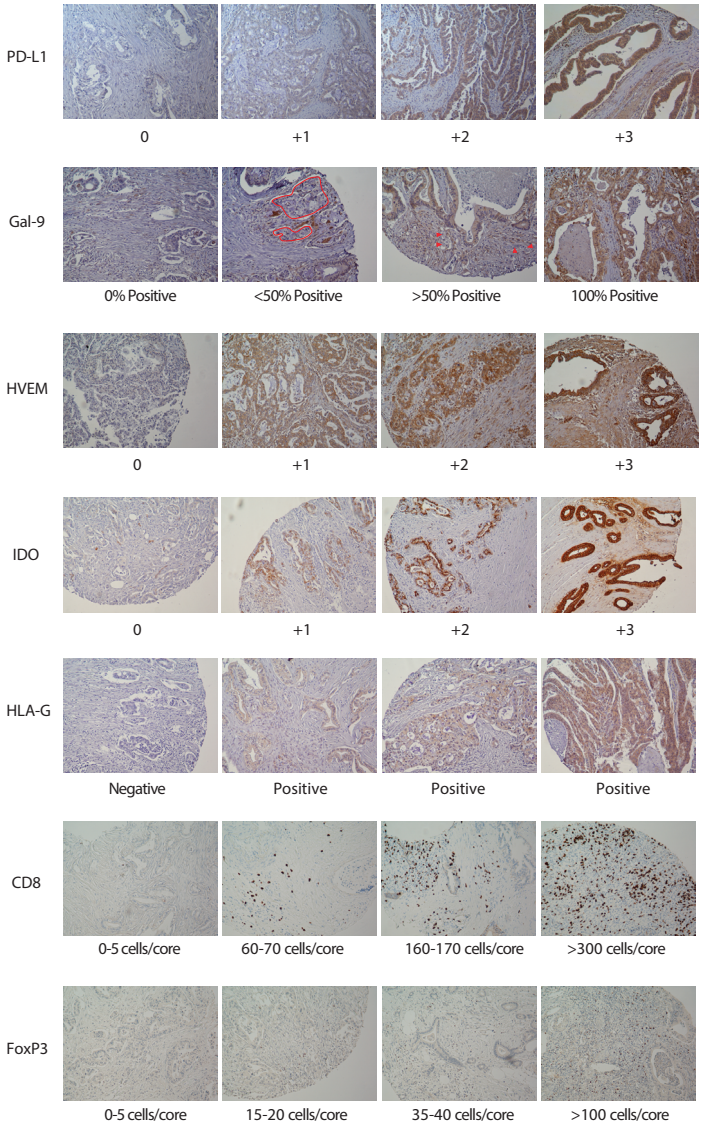

Figure 1. Representative stainings showing variable expression levels of immune inhibitory molecules and TIL on pancreas cancer cells. For PD-L1 and IDO there was little to no variability in the % of positive tumor cells per case thus only staining intensity was scored. Optimal cutoffs were a score of +1 or stronger for PD-L1 and a score of +2 or stronger for IDO. For Gal-9 and HVEM there was significant variability in the % of positive tumor cells thus both staining intensity and % of positive tumor cells was scored (pictures provided demonstrate this variability for Gal-9 only; red circled areas and arrows point to negatively stained tumor cells). Optimal cutoffs were a score of 0.4 (out of 3) for Gal-9 and a score of 1.7 (out of 3) for HVEM. For HLA-G relatively few cases showed expression at any level, thus any level of staining was considered positive. For CD8 and FoxP3 the number of positive cells were manually counted and averaged over the 5 tumor cores. Optimal cutoffs were for CD8: 142 TILs per core, for FoxP3: 25 TILs per core and for the CD8/FoxP3 ratio, a ratio of 6.5. Please also refer to the methods section for more information.

and IDO, where very little intra-core or inter-core heterogeneity was observed, only the intensity of cytoplasmatic staining was taken into consideration. IDO was considered high when the intensity score was +2 or stronger while PD-L1 was considered high when the intensity score was +1 or stronger (Figure 1). For HVEM and Gal-9 intra-core heterogeneity was observed, thus composite scores, including intensity and proportion of positive tumor cells, were calculated. Optimal cutoff values for cytoplasmatic HVEM staining was 1.7 (out of 3) and for Gal-9 it was 0.4 (out of 3). For HLA-G we detected staining in only 14.6% of cases, thus any case with positive staining was considered high and all negative cases were considered low. Finally the most optimal cutoffs for CD8 was 142 TILs per core, for FoxP3 was 25 TILs per core and for the CD8/FoxP3 ratio a ratio of 6.5.

Statistical analysis.

All analysis was performed in duplicate. Survival curves were estimated by the Kaplan-Meier method. Cancer-specific survival and recurrence-free survival were calculated from the date of surgery to the date of event (death from cancer or recurrence of cancer respectively). In case of no event patients were censored at last follow-up. The log-rank test was used to evaluate differences between survival curves of different groups. Optimal high vs low values were established by examining a grid of cutoffs and choosing the cutoff with the lowest -2 log likelihood. For multivariate analysis, the Cox proportional Hazard regression analysis was used with backward variable selection. Patients with missing values for the co-variates of interest were excluded from the statistical analysis. The associations between clinicopathologic parameters and the expression of immune inhibitory molecules, as well as the co-expression of the immune inhibiting molecules with each other, were examined using the χ^2 tests or the T-test as appropriate. For these associations a Bonferroni correction was applied to the p-values (0.05/8 immune biomarkers = 0.0063 for statistical significance). Internal validation was performed by generating 1,000 bootstrapped datasets. Sensitivity analysis was performed by excluding the patients who received neo-adjuvant treatment (n=4). In addition, further sensitivity analysis was performed by examining our findings between the *a priori* division of patients with cancers originating at the pancreas versus ampullary region. The statistical analysis was performed using the SPSS© 21 software.

RESULTS

Patients and baseline clinicopathologic characteristics.

Median survival was 17.5 months and median time to recurrence was 13.5 months. Baseline clinicopathologic characteristics can be seen in Table 1. The univariate hazard ratios for all the baseline clinicopathologic characteristics can be seen in Table 2, while

the respective Kaplan-Meier curves are depicted in Figure 2A. Similar associations with time to recurrence are shown in Supplementary Table 3 (univariate hazard ratios) and Supplementary Figure 2a respectively (Kaplan-Meier curves). In multivariate analysis of the clinicopathologic characteristics (Table 3a) margin status, lymph node status, tumor grade and pre-operative CA-19.9 level were independent predictors of cancer-specific survival, while T stage, tumor location (pancreas vs ampullary), use of systemic chemo, and age were not independent predictors of cancer-specific survival.

Table 3a Multivariate Cox proportional Hazard regression analysis of clinicopathologic parameters

Variables	HR	95% CI lower limit	95% CI upper limit	p-value
Margin status	2.02	1.36	3.00	**.001**
Lymph node status	2.77	1.76	4.36	**<.001**
Tumor differentiation	1.59	1.12	2.24	**.009**
CA-19.9 (log10)	1.31	1.00	1.71	**.047**
T stage	0.92	0.65	1.29	.613
Systemic chemotherapy	1.09	0.74	1.59	.676
Age	1.00	0.98	1.02	.714
Pancreas vs ampulla	0.98	0.63	1.51	.917

Table 3b Multivariate Cox proportional Hazard regression analysis of individual immune biomarkers adjusted for clinicopathologic characteristics*

Variables	HR	95% CI lower limit	95% CI upper limit	p-value
Clinicopathologic model + IDO	0.71	0.41	1.21	.205
Clinicopathologic model + **PD-L1**	0.55	0.35	0.87	**.010**
Clinicopathologic model + **Gal-9**	0.58	0.37	0.88	**.010**
Clinicopathologic model + **HVEM**	0.49	0.29	0.81	**.006**
Clinicopathologic model + HLA-G	0.53	0.28	1.01	.055
Clinicopathologic model + $CD8^+$TIL	0.77	0.50	1.17	.214
Clinicopathologic model + $FoxP3^+$TIL	0.85	0.58	1.25	.411
Clinicopathologic model + **CD8/FoxP3**	0.57	0.36	0.91	**.017**

* In Table 3b only the hazard ratios and 95%CI of the immune biomarkers adjusted for the clinicopathologic characteristics (clinicopathologic model) are shown. Margin status, lymph node status and tumor differentiation remained strong independent predictors of cancer-specific survival, in all cases, while T stage, age, the use of systemic chemotherapy and anatomic location (ampulla vs pancreas origin) remained non-significant in all cases, just as in Table 3a. CA-19.9 was significant, or bordered significance, in all cases.

Description of immune inhibitory molecule expression.

Figure 1 shows representative stainings for all molecules in cancer cells. At least some level of HVEM, PD-L1, Gal-9, IDO and HLA-G cancer cell cytoplasmatic staining was seen in 97.3%, 90%, 77%, 36%, and 14.6% of cases. Median $CD8^+$TIL number per tumor core was 116 (range 6-550) and median $FoxP3^+$TIL number per tumor core was 25 (range

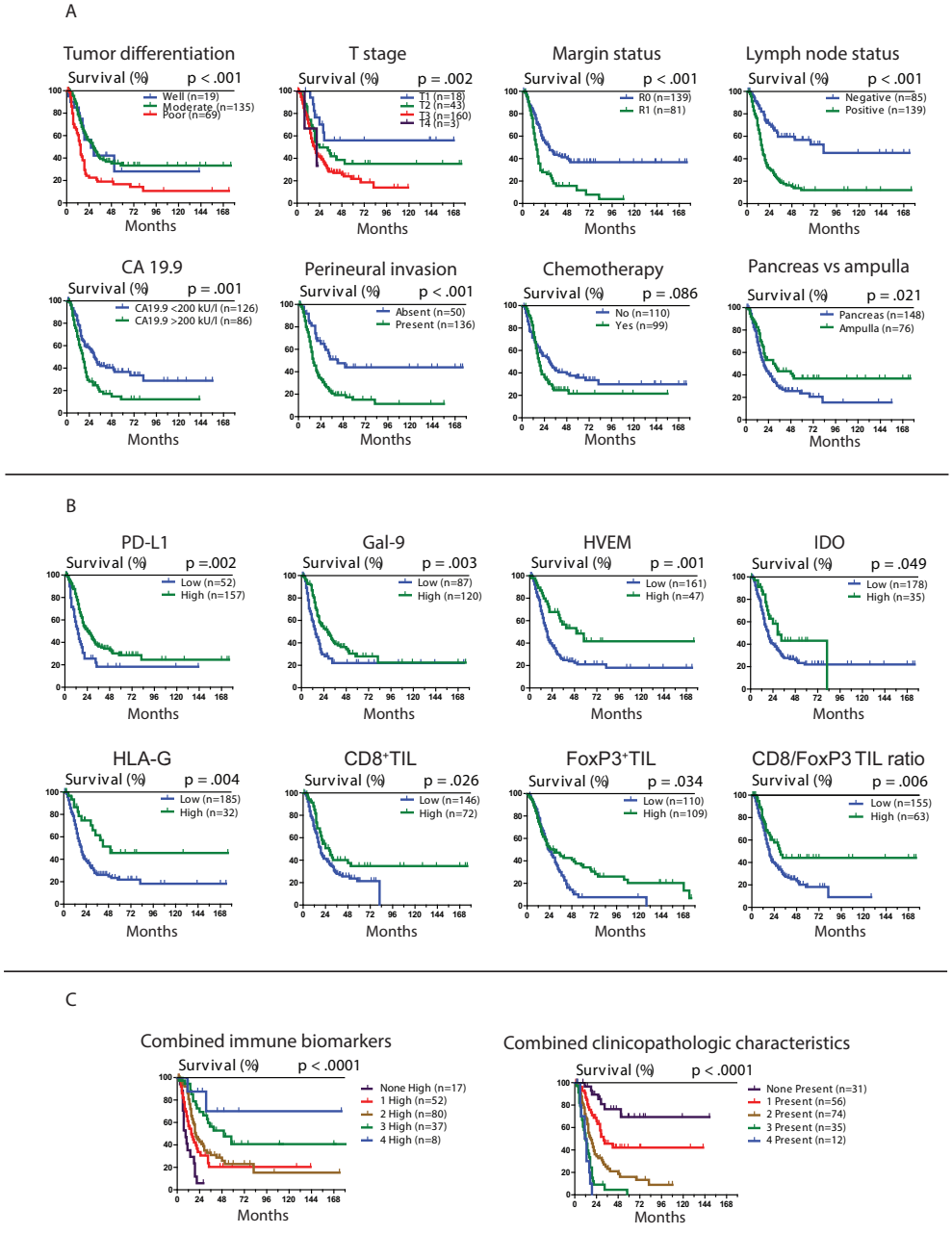

Figure 2. Pancreas cancer survival Kaplan-Meier graphs.
(A) Clinicopathologic characteristics. (B) Immune inhibitory molecules and TIL. (C) Combined immune biomarkers (PD-L1, Gal-9, HVEM and CD8/FoxP3 ratio) and combined clinicopathologic characteristics (margin status, lymph node status, tumor grade, CA19-9).

0-136). Supplementary Figure 3 shows the comparison between the two anti-PD-L1 antibodies (9A11 and 5H1) which was performed for selected cases. While the 5H1 antibody exhibited less intense staining, all positive cases with the 9A11 antibody were also positive with the 5H1 antibody, while all negative cases were negative with both antibodies without exception. Supplementary Figure 4 shows the expression of the immune inhibitory molecules in the TMA cores in comparison to their representative full section slides. This was done for a total of ten cases (four pairwise cases per antibody are shown). The expression patterns of all molecules in the TMA cores and their representative full slides were identical.

Associations of immune inhibitory molecule expression and TIL infiltration with clinicopathologic characteristics.

Cutoffs were optimized to produce the most discriminating biomarkers for cancer-specific survival and they are presented in the supplementary methods section. After Bonferroni correction it was found that high PD-L1 expression was associated with lower CA-19.9 ($p<.001$) and lower T-stage ($p=.002$), high Gal-9 was associated with lower tumor grade ($p=.004$), high HLA-G was associated with peri-neural invasion ($p=.001$) and high $CD8^+$TIL was associated with lower T-stage ($p<001$). In addition, PD-L1 expression was positively correlated with Gal-9 ($p<.001$), Gal-9 expression was positively correlated the CD8/FoxP3 ratio ($p=.005$) and HLA-G expression was positively correlated with HVEM ($p<.001$). Another notable association was between high PD-L1 and high $CD8^+$TIL ($p=.028$), which, despite not meeting the Bonferroni correction cutoff for significance, was nevertheless an *a priory* hypothesis [24].

Associations of immune inhibitory molecule expression and TIL with survival and recurrence.

Figure 2B shows the Kaplan-Meier curves for overall survival in relation to the optimized cut-offs of the immune inhibitory molecules and TIL under study and Table 2 shows the respected univariate hazard ratios. High cytoplasmatic expression levels of PD-L1, Gal-9, HVEM, IDO, and HLA-G as well as high $CD8^+$TIL and $FoxP3^+$TIL infiltration were associated with improved cancer-specific survival. Furthermore, an a *priori* hypothesis was that the ratio of $CD8^+$TIL to $FoxP3^+$TIL infiltration would predict better survival. Indeed, high CD8/FoxP3 ratio was associated with improved cancer-specific cancer survival (Figure 2B, Table 2). The results were similar for time to cancer recurrence (Kaplan-Meier curves in Supplementary Figure 2b, univariate hazard ratios in Supplementary Table 3).

Multivariate analysis.

We first examined the individual performance of each immune inhibitory molecule and TIL parameter adjusting for all the baseline clinicopathologic characteristics (Table 3b).

PD-L1, Gal-9, HVEM and the CD8/FoxP3-TIL ratio were each independent predictors of cancer-specific survival, while HLA-G showed a strong statistical trend. IDO, $CD8^+$TIL, and $FoxP3^+$TIL were not independent predictors of cancer-specific survival. We then investigated, in multivariate analysis, how all tested immune biomarkers and clinicopathologic characteristics behaved together in order to examine if the immune biomarkers were independent of each other (Table 4). Margin status, lymph node status, tumor grade, Gal-9, HVEM and CD8/FoxP3 ratio remained independent predictors of cancer-specific survival while PD-L1 (p=.062), and to a lesser degree HLA-G (p=.125), showed a strong trends towards such independence. IDO expression, age, systemic chemotherapy, T-stage, CA-19.9 and tumor location were not independent predictors of cancer-specific survival.

Table 4 Multivariate Cox proportional Hazard regression analysis of all immune biomarkers and all clinicopathologic characteristics*

Variables	HR	95% CI lower limit	95% CI upper limit	p-value
Margin status	2.39	1.56	3.64	**<.001**
Lymph node status	2.65	1.66	4.24	**<.001**
Tumor differentiation	1.50	1.04	2.16	**.030**
PD-L1	0.65	0.41	1.02	.062
Gal-9	0.62	0.40	0.97	**.038**
HVEM	0.49	0.28	0.87	**.014**
HLA-G	0.55	0.26	1.18	.125
CD8/FoxP3 ratio	0.51	0.29	0.87	**.014**

*Age, systemic chemotherapy, tumor location, T-stage, CA-19.9 (log10) and IDO are not independent. $CD8^+$TIL and $FoXP3^+$TIL were not individually included in the analysis due to the obvious interaction with the CD8/FoxP3 ratio and because they are not independent in Table 3b.

Co-expression patterns and survival.

Given the independent prognostic nature of the tested immune biomarkers, we investigated whether we could develop an even more informative predictive immunohistochemical biomarker panel. We chose to combine the immune biomarkers with the strongest prognostic ability, namely PD-L1, Gal-9, HVEM and the CD8/FoxP3-TIL ratio. Figure 2C (left panel), shows the Kaplan-Meier curve of the combined immune biomarker in relation to cancer-specific survival (HR 0.57 95%CI 0.47-0.69, p<.0001). In figure 2C (right panel), we contrasted these findings with the combined presence of the independently prognostic clinicopathologic characteristics, namely margin status, lymph node status, tumor differentiation and CA-19.9 (HR 2.08 95%CI 1.74-2.47, p<.0001). The ability of the immune biomarkers to prognosticate patients with resected pancreatic and ampullary cancers is comparable to the prognostication ability of known clinicopathologic characteristics.

Validation.

To internally validate our final results 1,000 bootstrapped datasets were generated. The combined immune biomarker remained significant during validation (HR 0.58 95%CI 0.47-0.73, p<.0001). Sensitivity analysis performed by excluding the patients who received neo-adjuvant therapy (n=4) did not significantly affect any of the univariate or multivariate analysis in our study. In addition, further sensitivity analysis was performed in the subgroups of cancers arising from the pancreas versus the ampullary region. Firstly, when looking into the individual immune biomarkers (Supplementary Table 4) it is apparent that the hazard ratio of each independent immune biomarker (PD-L1, Gal-9. HVEM, CD8/FoxP3 ratio) has comparable magnitude and the same direction in the pancreatic and ampullary cancer cohorts. Moreover, the hazard ratio of the combined immune biomarker for patients with pancreatic cancer was 0.62 (95%CI 0.48-0.79, p<.0001) and for patients with ampullary cancer was 0.52 (95%CI 0.37-0.72, p<.0001), thus of comparable magnitude and direction.

DISCUSSION

Pancreas cancer is one of the deadliest cancers known and therapeutic advances have been slow. We show, here, that multiple immune inhibitory molecules are expressed by pancreas and ampullary cancer cells and these molecules can, in principal, become interesting targets for immunotherapy. This is especially the case for PD-L1, Gal-9 and HVEM, and their respective receptors on immune cells, namely PD-1, TIM-3 and BTLA. Antibodies targeting these interactions are already in clinical practice for other cancer types [6], or are in preclinical and clinical development [25].

These immune inhibitory molecules have hardly been studied in relation to pancreatic cancer survival before. IDO has been shown to indeed be expressed in pancreas cancer, in 2 small studies [26, 27], however, the relationship of IDO expression to survival was not examined. PD-L1 expression and survival has been investigated in 2 small studies of 40 and 81 patients respectively [28, 29], while HLA-G expression has been examined in 3 studies, 2 of which have examined a relationship to survival (122 and 158 patients) [30-32]. Gal-9 and HVEM expression have never been studied in relation pancreatic cancer survival before.

We show that high expression of immune-inhibitory molecules and TIL counts are associated with better cancer-specific survival. However, while both high $CD8^+$TIL and $FoxP3^+$TIL counts are associated with better survival, it is only the CD8/FoxP3 ratio that is an independent predictor of cancer-specific death. In fact, a low CD8/FoxP3 ratio is one of the strongest independent predictors of cancer death in our study. One may question

why a high level of FoxP3$^+$TIL would be associated with better survival, since FoxP3$^+$ lymphocytes are known to be immunosuppressive and aid in cancer progression [7]. In fact, this same observation has been made in colorectal cancer [33]. One explanation is that FoxP3$^+$ regulatory T cells are recruited into tumors as a consequence of CD8+ T cell infiltration [34]. Thus, the presence of a FoxP3$^+$ infiltrate in a tumor simply signifies the concurrent presence of an active infiltrate of CD8$^+$TIL. It is only when the FoxP3$^+$TIL dominates that the prognosis becomes worse.

A similar argument can be made as to why high expression of PD-L1, Gal-9, HVEM and HLA-G are, counterintuitively, associated with better survival. One hypothesis is that their expression, especially the simultaneous expression of multiple molecules, is a signal of an active immunologic attack that is beneficial to patients with cancer. This phenomenon, called adaptive immune resistance, where immune inhibitory molecules are known to become overexpressed on cancer cells in response to IFN-γ and TIL infiltration, has been well described recently [24, 34-36]. The positive correlation between PD-L1 (p=.028) and HLA-G (p=.047) expression with numbers of CD8+ TIL and the positive correlation of Gal-9 (p=.005) expression with the CD8/FoxP3 ratio in our study supports the idea that expression of these molecules on pancreatic and ampullary cancer cells may be related to this phenomenon.

Interestingly, the prior studies on PD-L1 and HLA-G expression in pancreatic cancer have suggested the opposite; namely that high PD-L1 or high HLA-G expression are associated with poor survival [28-31]. One possible explanation for the differences is the scoring techniques used. While we focused our scoring on cancer cells, the study by Wang et.al. [29] used imaging software to examine total PD-L1 expression. This may include expression by stroma cells, TILs and especially expression on non-cancerous cells with atypia, which could certainly give different results. In our study we observed PD-L1 staining in non-neoplastic cells but were careful to distinguish neoplastic from non-neoplastic cells, prior to the TMA construction process. The respected photographs of PD-L1 expression in the Wang et. al. [29] study, but also in the Nomi et. al. study [28] (figures 1B and 1A in the respective publications) certainly raise the possibility of having included non-tumorous, atypical cells in the scoring methodology of these studies. Another explanation for the differences could be the use of non-validated antibodies. Both prior studies in pancreas cancer [28, 29] have used the MIH1 anti-PD-L1 clone for immunohistochemistry. This clone has been shown in multiple studies to not perform well when compared to other validated antibodies [37, 38]. The PD-L1 antibodies we used have been well validated [21, 39]. In the case of HLA-G, one prior study [32] used clone 4H84 which has been shown to be non-specific in binding its intended target [40, 41], a second study used a polyclonal antibody [31], while the third study did not provide information on the

antibody used [30]. Again, we used an HLA-G antibody that has been validated in previous studies [42, 43]. In addition, we provide the expected positive control staining in placenta tissue for both PD-L1 and HLA-G (Supplementary Figure 1), which was not provided by the above mentioned studies in either PD-L1 [28, 29] or HLA-G [31, 32, 41].

We show that the expression of these immune inhibitory molecules can, in combination, form clinically relevant biomarkers. The independent nature of these immune biomarkers means that they influence prognosis irrespective of traditional clinicopathologic characteristics, such as lymph node metastasis or margin status. In our cohort, while every additional independent adverse clinicopathologic characteristic doubled the risk of cancer death, the presence of high levels of each additional immune biomarker reduced that risk to nearly half. This indicates the potential of our immune biomarker panel to aid in the prognostication of patients with pancreatic and ampullary cancers. While further standardization of the staining techniques is required for clinical development, tumor tissue from resected patients is readily available and immunohistochemistry is a standard, cost effective, clinical laboratory technique.

PD-L1 expression has already emerged as a biomarker of response to anti-PD-1 antibodies [6]. However, PD-L1, as a single biomarker, is not perfect. In melanoma, anywhere from 6-41% of patients who are considered PD-L1 negative do actually respond to anti-PD-1 antibodies, while half or more of the patients considered to be PD-L1 positive, do not respond [44]. It is likely that a broader immune biomarker panel, one which takes into consideration multiple immune inhibitory molecules, as well as the immune lymphocytic infiltrate, would lead to improved prediction of the patients likely to benefit from immune checkpoint inhibitors. Thus, we propose incorporation of such biomarker panels in prospective immunotherapy clinical trials.

In addition, in the case of PD-L1, an ongoing issue is the proper histological evaluation of PD-L1 expression. Both cytoplasmatic and membranous PD-L1 staining have been observed in various cancers before [24, 45, 46] and we, in our study, observed predominately cytoplasmatic staining. It has recently been hypothesized that cytoplasmatic tumor staining represents intracellular stores of PD-L1 ready to be transported to the membrane upon contact with immune cells [24]. In fact this phenomenon is well demonstrated for another immune inhibitory molecule expressed on T-cells, namely CTLA-4 [47, 48]. While debate is ongoing, as to the respective importance of cytoplasmatic versus membranous PD-L1 staining in the various cancer types [24], it is likely that cytoplasmatic PD-L1 expression is biologically important and should not be ignored.

We show that the results of the expression of the immune biomarkers do not substantially differ between pancreatic and ampullary cancers (Supplementary Table 4). In addition, in multivariate analysis, the hazard ratios for the combined immune biomarker are of similar magnitude and direction in the pancreatic and ampullary cohorts. Thus our results are not cancer specific. In addition, tumor location (pancreas vs ampulla) is not an independent predictor of cancer-specific survival (Table 3a) or cancer recurrence (Supplementary Table 3). Thus, it does not appear that cancers arising from the ampulla behave biologically different than cancers arising from the pancreas, when correcting for adverse clinicopathologic factors. However, we cannot generalize our findings to a broader definition of peri-ampullary tumors, which frequently include duodenal cancers and distal cholangiocarcinomas, since we have excluded these tumors from our cohort. As a result, while our study provides information on the immunologic profile of ampullary cancers, a type of cancer that rarely attracts research attention, further work is needed to immunologically characterize all different kinds of peri-ampullary tumors.

Our study has several strengths. This is the first study to examine the relationship of the expression of Gal-9 and IDO with survival in pancreatic and ampullary cancer and the largest study to, similarly, examine the expression of PD-L1 and HLA-G. In addition, the examination of multiple immune biomarkers in a single, large, homogeneous cohort is a novel approach, which allows for evaluating the interactions between these biomarkers. Furthermore, the size of our cohort allowed for confident internal validation of our main results by bootstrapping. This method has been shown to be the preferred internal validation method of such datasets [49]. Additional strengths are the successful use of TMAs in our study. We used five 1-mm cores to construct our TMAs which resulted in a substantial amount of cancer cells to evaluate. This is despite the fact that pancreas cancer is generally associated with significant amounts of stroma tissue. We also show that expression of these molecules in full slides were in complete agreement with our TMA findings (Supplementary Figure 4). Thus, the use of TMAs in assessing tumor expression patterns of these molecules, in pancreas cancer, is possible.

However, our study has also limitations. We had limited potential to systematically examine the surrounding peri-tumoral tissue. While general observations could be made, we were not able to systematically examine the non-neoplastic cells. In addition, our choice of immune biomarkers studied is not exhaustive. As our understanding of tumor immunology improves, additional molecules may become interesting to study.

In conclusion, pancreas and ampulary cancers are rich in expression of immune inhibitory molecules. High cancer cell expression of PD-L1, Gal-9, HVEM and HLA-G, and high CD8/FoxP3 TIL ratio are associated with improved cancer-specific survival. The

combination of such immune biomarkers can be a powerful prognostication tool for these patients. Such panels of immune biomarkers should be considered for inclusion in future immunotherapy clinical trials.

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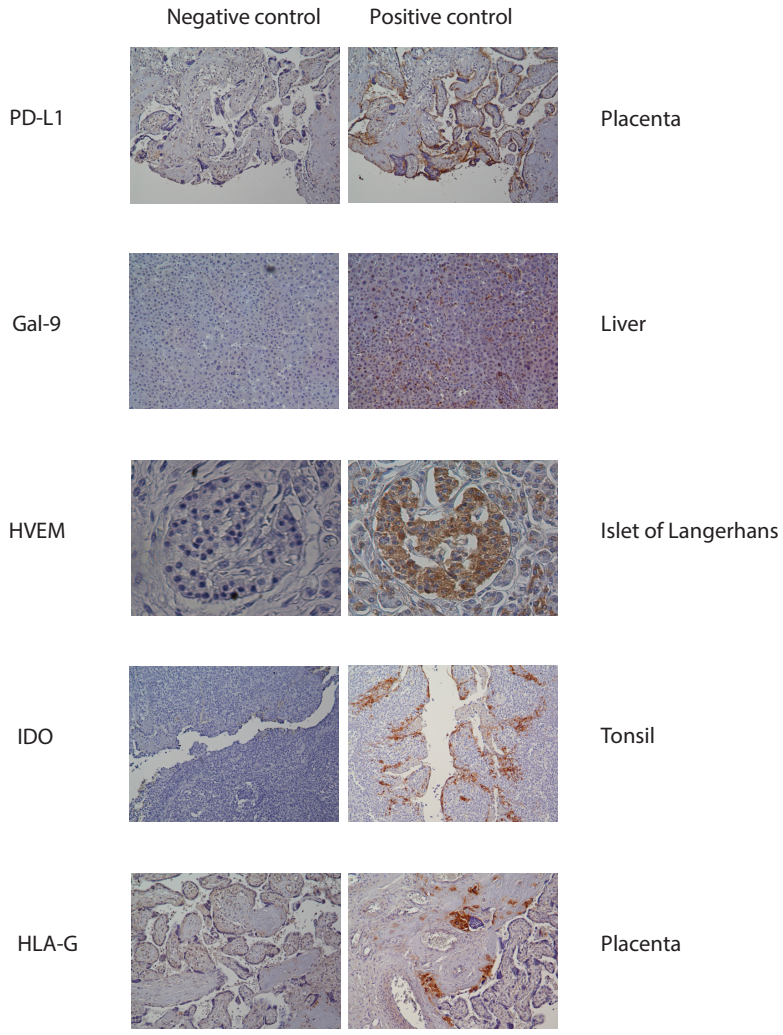
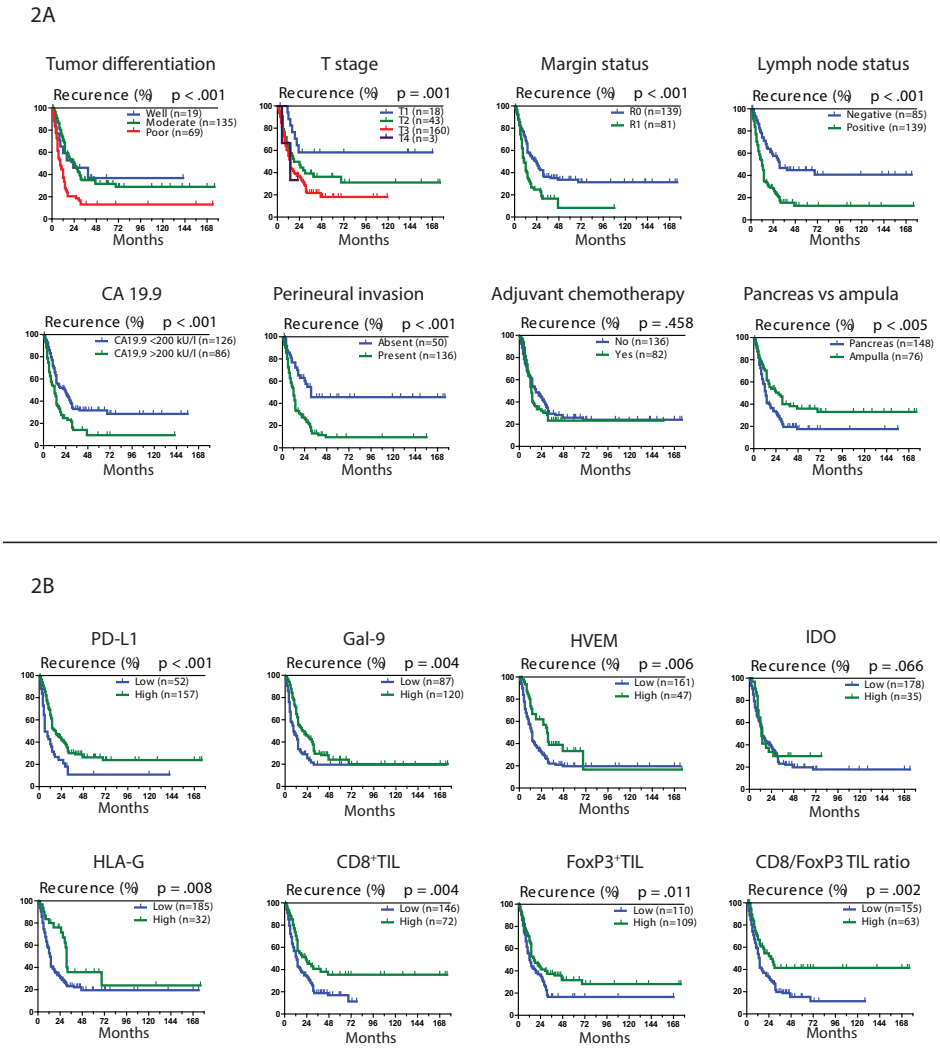
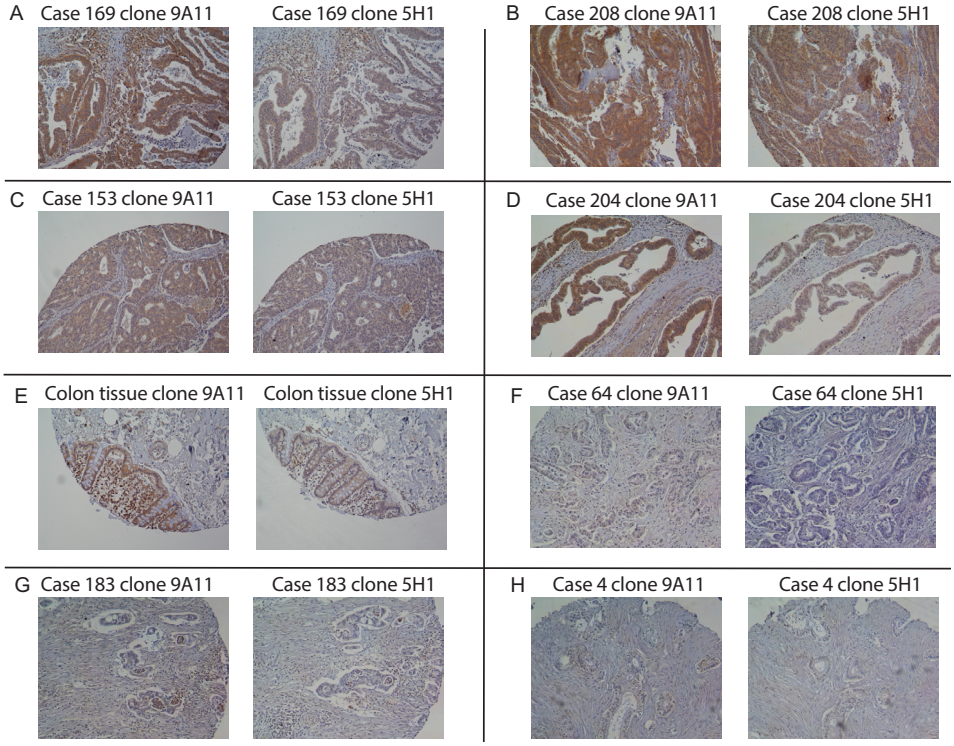
Supplementary Figures



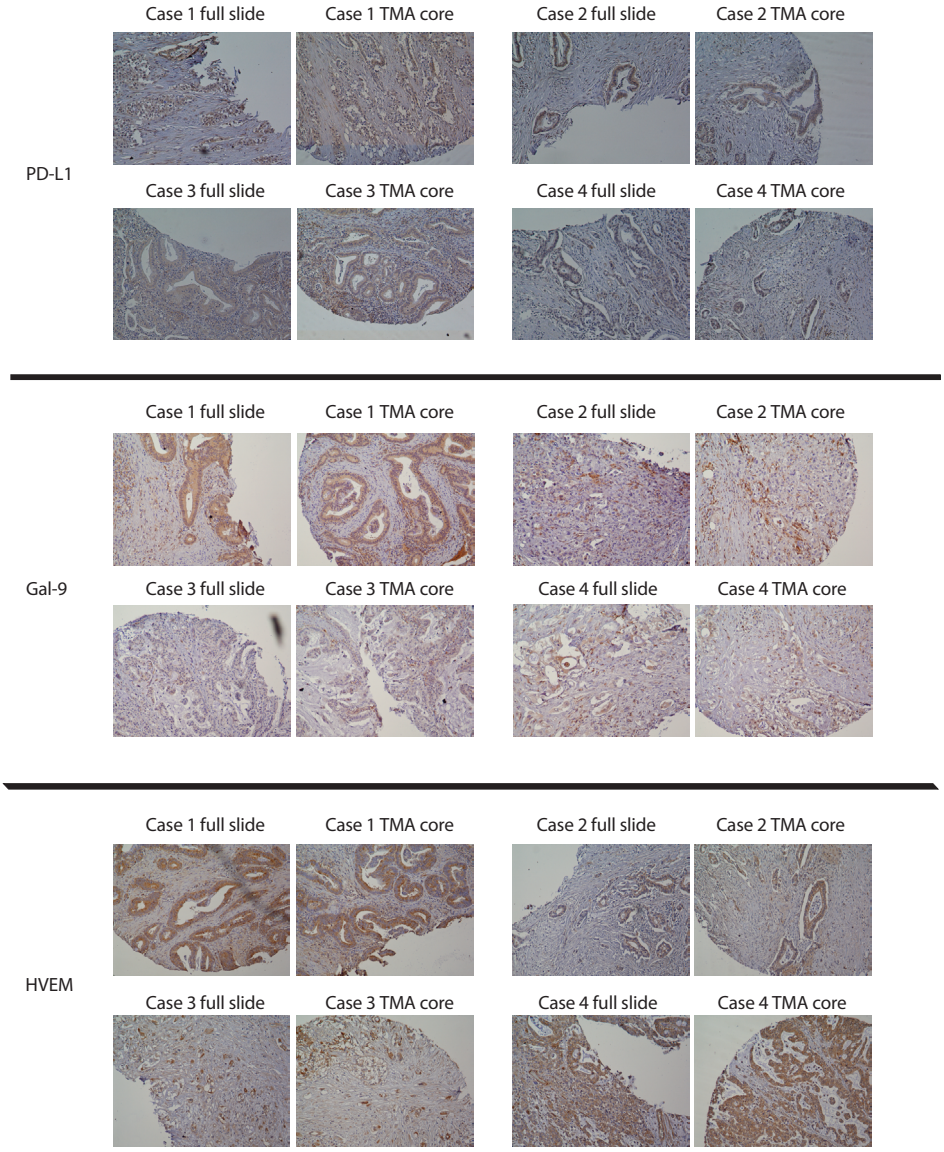
Supplementary Figure 1. Positive and negative control tissues.
PD-L1 stains the microvillous membrane in placenta tissue as described by:
Velcheti V, et al, Lab Invest (2014) 94, 107-116.
Gal-9 stains kupffer cells but not hepatocytes in healthy liver in accordance with:
Mengshol JA, et al, PlosONE (2010) 5, e9504
HVEM stains islets of Langerhans in pancreas tissue in accordance with:
http://www.proteinatlas.org/ENSG00000157873-TNFRSF14/tissue/pancreas
IDO stains lymphocytes in tonsil lymphoid tissue underlying the mucosal epithelium in accordance with
Munn DH, et al, Science (2002) 297, 1867-1870.
HLA-G stains exlusively extravillous trophoblastic cells in placenta tissue in accordance with:
Apps R, et al, Trends Immunol (2008) 29, 313-321.



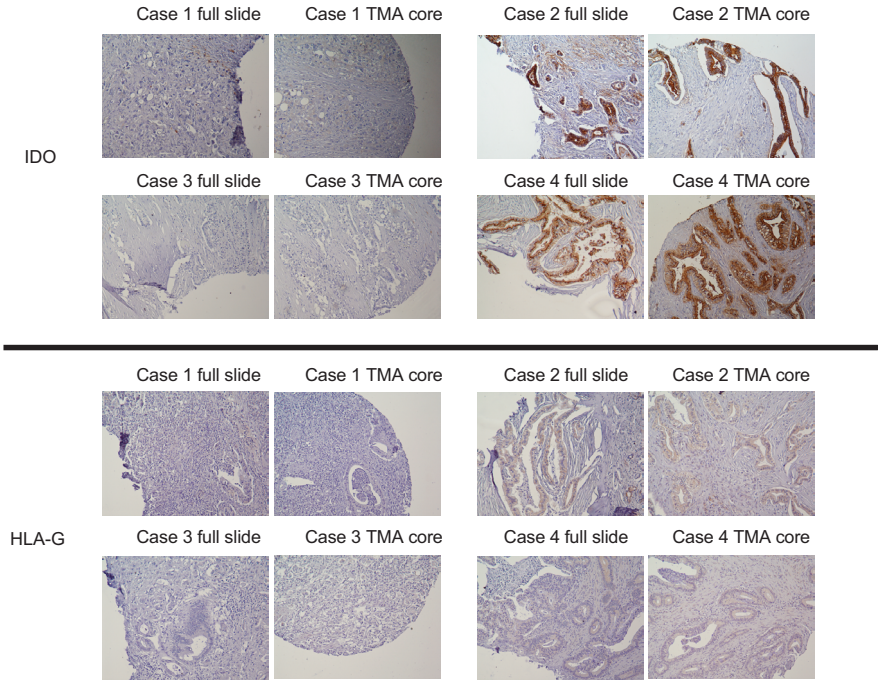
Supplementary Figure 2. Time to recurrence Kaplan-Meier curves. (A) clinicopathologic characteristics, (B) immune biomarkers.



Supplementary Figure 3. Representative PD-L1 stainings using two validated anti-PDL1 antibodies. (A-D) Positive tumor cases. (E) Normal colon tissue shows similar staining patern with both antibodies. (F-H) Negative tumor cases. Note in G that while tumor cells are negative with both antibodies, TIL cells are similarly positive.



Supplementary Figure 4. Side by side comparison of TMA cores and full slides.
Four representative pairs are shown per antibody. For IDO and HLA-G see next page



Supplementary Figure 4 (continued). Side by side comparison of TMA cores and full slides. Four representative pairs are shown per antibody.

Supplementary tables

Supplementary Table 1. Tumor location and histology

Location	Number of patients (%)
Pancreas head	122 (54.5)
Pancreas body	10 (4.5)
Pancreas tail	16 (7.1)
Ampulla of Vater	76 (33.9)

Histology	Number of patients (%)
Adenocarcinoma	214 (95.5)
Adeno-squamous	4 (1.8)
Acinus-cell	3 (1.3)
Anaplastic	3 (1.3)

Supplementary Table 2. Primary antibodies used

Antigens	Antibody source	Clone	Retrieval buffer	Dilution
PD-L1	G. Freeman[a]	9A11	Tris EDTA	1:50
PD-L1	Haidong Dong[b]	5H1	Tris EDTA	1:50
Gal-9	R&D systems[c]	Goat polyclonal	Tris EDTA	1:200
HVEM	Millipore[d]	2G6-2C7	Citric acid	1:200
IDO	Millipore[e]	10.1	Citric acid	1:200
HLA-G	Exbio[f]	MEM-G1	Citric acid	1:200
CD-8	Ventana[g]	SP-57	CC1[h]	Ready to use
Fox-P3	e-bioscience[i]	236A/E7	CC1[h]	1:100

[a] Kindly provided by Dr. Gordon J Freeman, Dana-Farber Cancer Institute, Boston, MA (Mahoney KM, et.al., Cancer Immunol Res 2015; 3: 1308-1315).

[b] Kindly provided by Dr. Haidong Dong, Mayo Clinic, Rochester, MN (Dong H, et.al., Nat Med 2002; 8: 793-800)

[c] http://www.rndsystems.com/Products/AF2045 (Mengshol JA, et.al., PLoS One 2010; 5: e9504)

[d] http://www.merckmillipore.com/NL/en/product/Anti-TNFRSF14-Mouse-mAb-%282G6-2C7%29,EMD_BIO-AP1159?CategoryName=000000260002b67900020023&CategoryDomainName=Merck-MerckMillipore

[e] http://www.merckmillipore.com/NL/en/product/Anti-Indoleamine-2%2C3-dioxygenase-Antibody%2C-clone-10.1,MM_NF-MAB5412?bd=1#documentation (Soliman H, et.al., Cancer Immunol Immunother 2013; 62: 829-837)

[f] http://www.exbio.cz/products/clone.py?idclone=CLO000000000000078 (Boyson JE, et.al., Proc Natl Acad Sci U S A 2002; 99: 16180-16185)

[g] http://ventana.com/product/33?type=28

[h] Cell Conditioning Solution (CC1 Ventana Ref.: 950-124)

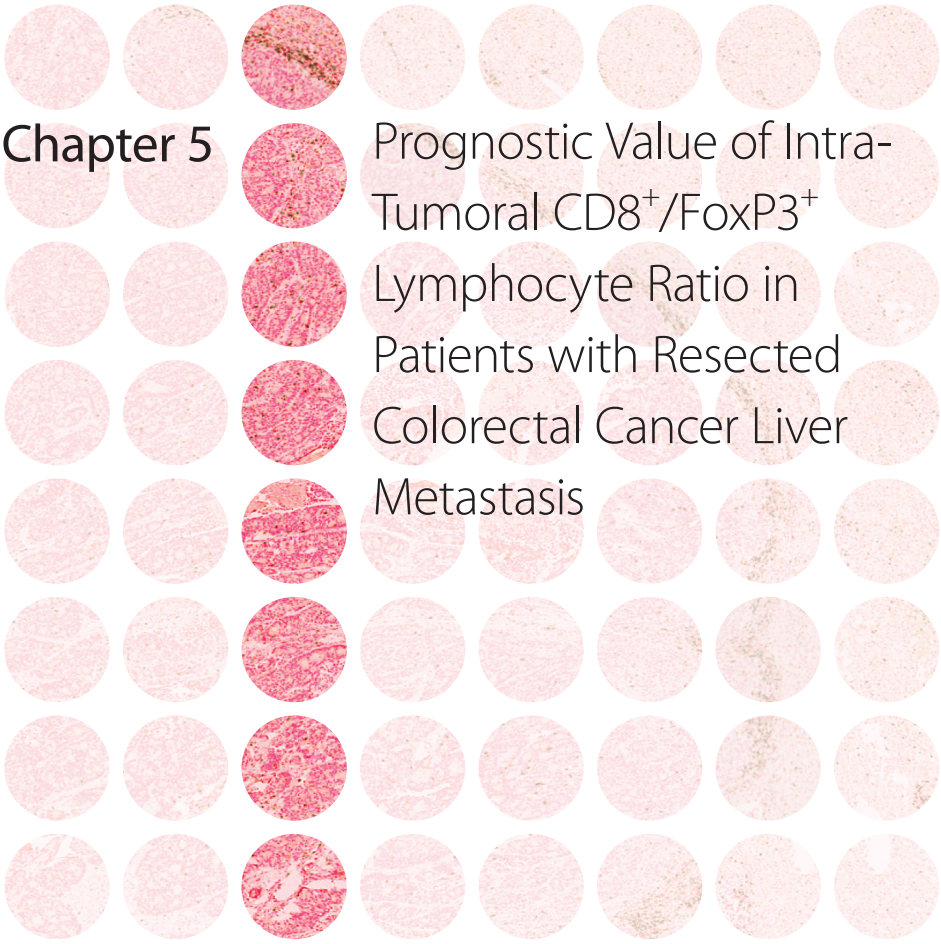
[i] http://www.ebioscience.com/human-foxp3-antibody-purified-236a-e7.htm (Banham AH et.al., Vet Immunol Immunopathol 2009; 127: 376-381)

Supplementary Table 3. Univariate Cox proportional Hazard regression analysis of patients' recurrence-free survival

Variables	HR	95% CI	p-value
Tumor differentiation	1.89	1.40-2.56	**<.001**
T stage	1.64	1.23-2.01	**.001**
Margin status	2.03	1.44-2.84	**<.001**
Lymph node status	2.52	1.74-3.65	**<.001**
CA-19.9 log(10)	1.48	1.17-1.86	**.001**
Peri-neural invasion	2.73	1.72-4.32	**<.001**
Pancreas vs ampulla	1.66	1.16-2.38	**.005**
Adjuvant chemotherapy	1.14	0.81-1.59	.458
Age	0.99	0.98-1.01	.410
IDO	0.64	0.39-1.03	**.066**
PD-L1	0.50	0.35-0.73	**<.001**
Gal-9	0.60	0.43-0.85	**.004**
HVEM	0.56	0.37-0.85	**.006**
HLA-G	0.51	0.30-0.84	**.008**
CD8-TIL	0.58	0.40-0.84	**.004**
FoxP3-TIL	0.65	0.47-0.91	**.011**
CD8/FoxP3 ratio	0.52	0.35-0.80	**.002**

Supplementary Table 4. Univariate Cox proportional Hazard regression analysis of patients' Cancer-specific survival

	Full cohort (as in Table 2)			Pancreas cancer			Ampulla cancer		
Variables	HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
Differentiation	1.79	1.32-2.43	**<.001**	1.59	1.11-2.26	**.011**	2.10	1.15-3.84	**.016**
T stage	1.57	1.19-2.09	**.002**	1.29	0.90-1.84	.168	1.82	1.18-2.84	**.007**
Margin status	2.12	1.56-3.13	**<.001**	2.05	1.35-3.13	**.001**	1.99	0.91-4.32	.084
Lymph node status	3.24	2.16-4.85	**<.001**	3.36	2.03-5.57	**<.001**	3.04	1.53-6.07	**.002**
CA-19.9 log(10)	1.49	1.18-1.95	**.001**	1.54	1.02-2.33	.055	1.33	0.99-1.79	**.014**
Peri-neural invasion	2.46	1.55-3.89	**<.001**	1.35	0.71-2.53	.359	3.67	1.76-7.66	**.001**
Pancreas vs ampulla	1.54	1.06-2.22	**.021**	NA	NA	NA	NA	NA	NA
Any chemotherapy	1.36	0.96-1.92	.086	1.20	0.79-1.84	.391	1.34	0.70-2.57	.381
Age	1.00	0.98-1.03	.910	1.00	0.80-1.02	.792	1.00	0.97-1.03	.913
IDO	0.58	0.35-1.00	**.049**	0.42	0.21-0.84	**.014**	1.02	0.45-2.30	.963
PD-L1	0.54	0.36-0.79	**.002**	0.59	0.38-0.94	**.025**	0.49	0.23-1.03	.060
Gal-9	0.58	0.41-0.83	**.003**	0.57	0.37-0.88	**.011**	0.58	0.32-1.08	.087
HVEM	0.45	0.28-0.72	**.001**	0.50	0.28-0.89	**.018**	0.40	0.18-0.91	**.028**
HLA-G	0.43	0.24-0.76	**.004**	0.66	0.29-1.51	.324	0.38	0.17-0.87	**.021**
CD8-TIL	0.65	0.44-0.95	**.026**	0.67	0.40-1.14	.136	0.77	0.42-1.41	.397
FoxP3-TIL	0.69	0.49-0.97	**.034**	0.79	0.50-1.15	.197	0.67	0.36-1.25	.209
CD8/FoxP3 ratio	0.55	0.36-0.84	**.006**	0.67	0.41-1.10	.115	0.40	0.17-0.89	**.026**



Chapter 5

Prognostic Value of Intra-Tumoral $CD8^+/FoxP3^+$ Lymphocyte Ratio in Patients with Resected Colorectal Cancer Liver Metastasis

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Under Submission

ABSTRACT

Introduction: Patients with isolated colorectal cancer liver metastases frequently undergo metastatectomy with curative intent. Biomarkers selecting patients for surgical intervention are needed. Tumor infiltrating lymphocytes (TIL) have shown significant prognostic potential in the setting of primary colorectal cancer, however, their role in the setting of colorectal cancer liver metastasis is poorly studied. In our study, we quantified tumor infiltrating CD8+ cytotoxic T-cells and Foxp3+ regulatory T-cells in relation to their spatial distribution at the metastatic site in the liver and examined their association with recurrence and survival.

Methods: TILs were isolated from fresh tumor tissues of 47 patients undergoing resection for colorectal cancer liver metastasis. Archived paraffin embedded tissue was retrospectively retrieved from the same patients. Patient clinicopathologic characteristics and follow up information were retrieved from the electronic records. Immunohistochemistry on full tissue sections was used to examine the presence of $CD8^+$ and $FoxP3^+$ cells both in the intra-tumoral and the peri-tumoral compartments. Flow cytometry was used to measure the proportions of cytotoxic T-cells ($CD8^+$) and regulatory T-cells ($CD4^+$ $CD25^+$ $FoxP3^+$) within $CD45^+$ TIL.

Results: By immunohistochemistry, individual concentrations of intra-tumoral or peri-tumoral $CD8^+$ and $FoxP3^+$ cells were not prognostic of colorectal cancer survival. However, the intra-tumoral, but not the peri-tumoral, $CD8^+/FoxP3^+$ ratio was an independent predictor of survival (HR 0.31, 95% CI 0.12-0.79, p=.0314) in multivariate analysis. By flow cytometry the intra-tumoral $CD8^+$/regulatory T-cell ratio was also found to be an independent predictor of survival (HR 0.45, 95% CI 0.20-0.99, p=.044). From the clinicopathologic characteristics only the lymph node status of the primary cancer was prognostic of survival. (HR 2.48, 95% CI 1.10-5.60, p=.024).

Conclusion: The ratio of cytotoxic ($CD8^+$) to regulatory ($FoxP3^+$) T-cells, in the intra-tumoral compartment but not in the peri-tumoral compartment, can predict survival after resection of colorectal liver metastases.

INTRODUCTION

Colorectal cancer is the third leading cause of cancer related death worldwide and the liver is the most common site of metastatic disease [1]. Unlike other solid tumors it is now standard for patients with isolated colorectal cancer liver metastases (CRCLM) to be considered for resection upon presentation. In fact, 20% of patients with CRCLM are resected with curative intent [2]. However, there is a wide range of clinical outcome following hepatic resection, ranging from cancer recurrence and death soon after resection to long-term cure from the disease. While clinical characteristics, such as the clinical risk score (CRS) [3], can prognosticate patients undergoing resection for CRCLM, the prognostication of these patients is far from ideal. New biomarkers to potentially select these patients for resection are needed.

The immune system plays an important role in cancer surveillance and elimination [4]. Tumor infiltrating lymphocytes (TILs) have emerged as potential biomarkers in various cancers [5]. In primary, non-metastatic, colorectal cancer the presence of TILs represent a robust prognostic biomarker that is superior to known clinicopathologic characteristics [6]. Multiple studies have now shown that TILs are associated with improved survival in primary colorectal cancer [7-9]. The hypothesis stemming from these observations is that an immune infiltrate, at the tumor site, represents an active immunologic attack against the cancer, which in turn is associated with a better prognosis. A similar relationship between TILs and survival has been investigated in the setting of CRCLM. TILs have generally been associated with improved outcome in patients with resected CRCLM [10-12].

However, TILs have been heterogeneously defined in the literature. It is known that the quality of the immune infiltrate is important since different T-cell subsets have opposite effects in the tumor microenvironment [13]. $CD8^+$ T-cells represent cytotoxic lymphocytes with antitumor properties. On the other hand $CD4^+CD25^+FoxP3^+$ T-cells represent T-regulatory cells (Tregs) which are known to have immunosuppressive and tumor supporting properties. While increased numbers of intra-tumoral $CD8^+$ effector T-cells are almost always associated with improved prognosis, there are contradictory findings as to the prognostic significance of intra-tumoral Tregs, since some studies find them associated with improved prognosis while others with worse prognosis, in various cancers [14].

Another source of heterogeneity in prior studies is the histologically uneven distribution of TILs at the tumor site. The majority of TILs appear to concentrate in the peri-tumoral area, sometimes not in direct contact with tumor cells. Only a minority of TILs penetrate to the tumor tissue itself. It is unclear what the relative significance of these TIL cell populations are.

The aim of the current study was to examine to composition and spatial distribution of TILs in CRCLM and to relate the TIL findings to prognosis. We examined the composition of TILs by quantifying numbers of $CD8^+$ effector T-cells and Tregs. We examined the spatial distribution of TILs by using full tissue slides to examine TIL populations in the peri-tumoral and tumoral areas separately. We hypothesized that TIL penetration in the tumoral area will be more prognostic than TIL populations present in the peri-tumoral area, and that the ratio of immune effector ($CD8^+$) to immune suppressive (Tregs) T-cells will be more prognostic than the concentrations of each individual cell type alone. We used and compared the results from two different techniques, namely immunohistochemistry on paraffin embedded tissues and flow cytometry on TILs freshly isolated from tumor tissues.

PATIENTS AND METHODS

Patient population and tissue samples.

A total of 47 patients who underwent hepatic resection for CRCLM at the Erasmus University MC Cancer Institute between September 2009 and November 2011 were enrolled in our study. Fresh liver tumor tissue was used to isolate TILs. Formalin fixed paraffin-embedded tissue from these same patients was retrieved from the pathological archive. Baseline clinicopathologic characteristics as well as follow-up information on cancer recurrence and death were retrospectively collected from the electronic patient records. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Medical Ethical Committee of Erasmus MC.

Clinical risk score.

The Clinical Risk Score was calculated according to Fong at. al., [3]. The presence of any one of the following five parameters was given a score 1 while the absence a score of 0: node-positive primary, disease free interval from primary to metastases < 12 months, number of hepatic tumors >1, largest hepatic tumor >5 cm and carcinoembryonic antigen level > 200 ng/ml.

Immunohistochemistry.

4µm thick sections were mounted on Superfrost PlusTM slides. For the stainings a Ventana Benchmark Ultra automated staining system (Ventana Medical System, Tuscon, AZ, USA) was used, in a clinical laboratory setting. For CD8, the sectioned specimens were processed for 16 min antigen retrieval using Cell Conditioning Solution (CC1 Ventana Ref.: 950-124). After 32 min incubation with the primary antibody (clone SP-57) at 36 °C and amplification with Optiview amplification kit (Ventana ref.:760-099), detection and

visualization was done with OptiView Universal DAB detection kit (Ventana Ref.: 760-700). The sections were counterstained with hematoxylin II (Ventana Ref.: 790-2208). For Fox-P3 the same protocol was applied with the exception the sectioned specimens were processed for 64 min antigen retrieval, the primary antibody (clone 236A/E7) dilution was 1:100, and amplification was performed with the Ultraview amplification kit (Ventana ref.:760-080). Figure 1 shows representative stainings for CD8 and FoxP3 as well as positive and negative control tissues.

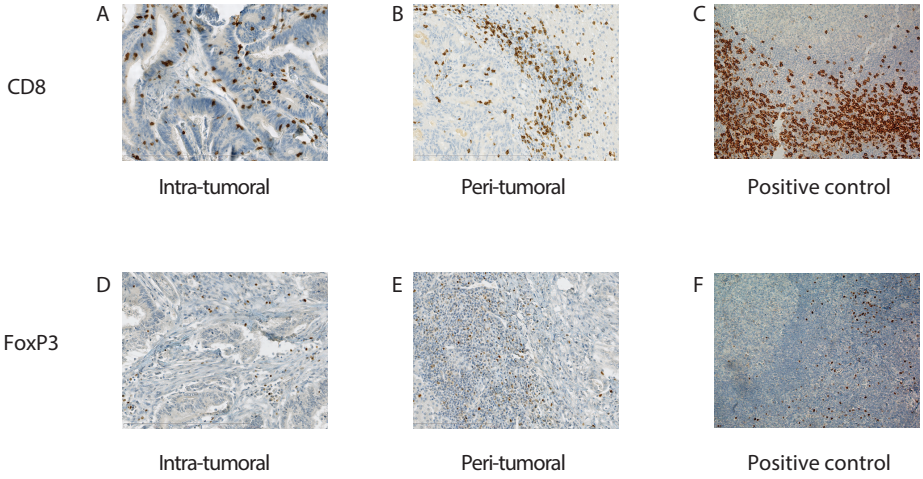
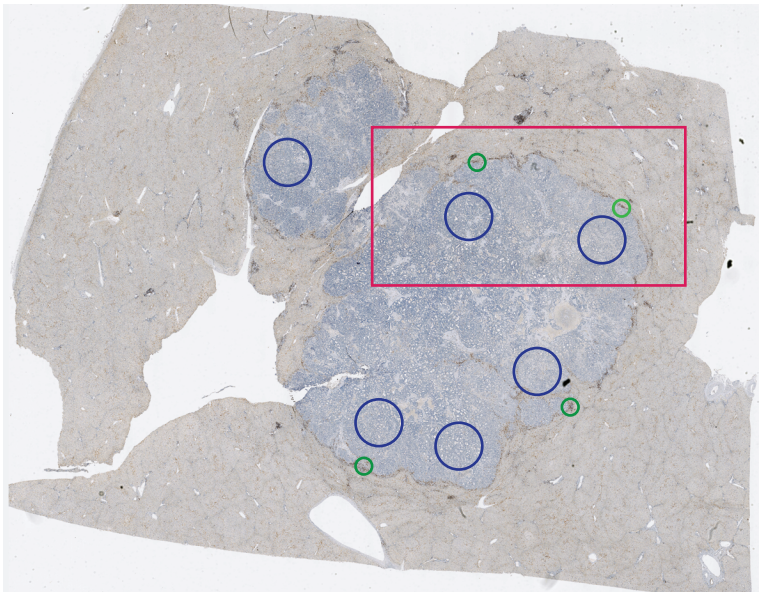


Figure 1. Representative CD8 and FoxP3 immunohistochemical stains. A) Intra-tumoral and peri-tumoral $CD8^+$ cells in CRCLM. B) $CD8^+$ cells in positive control tonsil tissue. C) Negative control tonsil tissue. D) Intra-tumoral and peri-tumoral $FoxP3^+$ cells in CRCLM. E) $FoxP3^+$ positive cells in positive control tonsil tissue. F) Negative control tonsil tissue.

Evaluation of immunohistochemistry staining.

Stained full tumor slides were electronically scanned using NanoZoomer 2.0HT (Hamamatsu Photonic, Shizuoka, Japan) at a magnification of 40x. Automated digital image analyses was performed using the Visiopharm Integrator System (Visiopharm, Hoersholm, Denmark). In each case 4-6 circular areas with a diameter of 0.54 mm (area = 1 high power field), from either the intra-tumoral area or the peri-tumoral area, were used to measure the concentrations of $CD8^+$ cells and $FoxP3^+$ cells. For the peri-tumoral areas, the areas used for analysis were centered the middle of the peri-tumoral infiltrate. For the intra-tumoral areas, the areas used for analysis were close to the tumor border in order to avoid necrotic areas towards the center of the tumor. A representative image used for analysis can be found in Figure 2. $CD8^+$ and $FoxP3^+$ cell concentrations were measured as cells/mm^2. All cases were visually reviewed to ensure accuracy of the measurements.

A



B

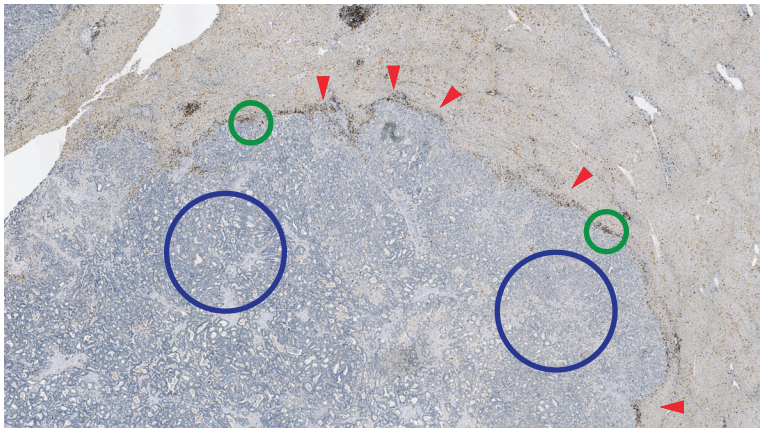


Figure 2. Representative example of areas used for analysis of CD8$^+$ and FoxP3$^+$ cell concentrations. A) Photograph of full slide. B) Zoom-in on red square of picture 2A. Blue circles represent intra-tumoral areas used for analysis. Green circles represent peri-tumoral areas used for analysis. The green circular areas are 0.23 mm^2 or 1 HPF. Note that the areas used for peri-tumoral analysis are centered on the middle of the peri-tumoral infiltrate. Red arrows indicate the peri-tumoral infiltrate.

TIL isolation:

Single cell TILs were isolated from tumor tissue via tissue digestion as previously described [15-17]. Briefly, fresh tumor tissue was cut into small pieces and digested with 0.5 mg/mL of collagenase (Sigma-Aldrich, St. Louis, MO) and 0.1 mg/mL of DNase I (Roche,

Indianapolis, IN) for 30 minutes at 37 °C. Cell suspensions were filtered through cell strainers and mononuclear cells (MNCs) were obtained by Ficoll density gradient centrifugation. Viability was determined by trypan blue exclusion.

Flow Cytometry Analysis

Mononuclear cells isolated from tumor tissue were analyzed for expression of surface and intracellular markers using the following anti-human antibodies: PerCP-labeled anti-CD8 (SK-1) and APC-H7-labeled anti-CD4 (SK3) from BD Biosciences; APC-labeled anti-FoxP3 (PCH101), PeCy7-labeled anti-CD3 (UCTH1), eFluor®450-labeled anti-CD45 (HI30) and eFluor®450-labeled anti-CD25 (BC96) from e-biosciences and anti CD56 (NHK-1) from Beckman Coulter. Cells were incubated with the antibodies 30 min at 4 °C in the dark, then washed and fixed with 1% paraformaldehyde. For intracellular cytokine staining, cells were incubated with the FoxP3 antibody using the nuclear staining buffer set from e-biosciences. Dead cells were excluded by using the LIVE/DEAD fixable dead cell stain kit with aqua fluorescent reactive dye (Invitrogen). Cells were analyzed in a FACSCanto II system (BD Biosciences, San Diego, CA). The gating strategy has been previously described [15]. Numbers of $CD8^+$ TILs were defined as the proportion of $CD8^+$ cells within live $CD45^+$ live cells, while numbers of T-regulatory cells were defined as the proportion of $CD4^+$ $CD25^+$ $FoxP3^+$ within live $CD45^+$ cells.

Statistical analysis.

All analyses were performed in duplicate. Cancer-specific survival and recurrence-free survival were calculated from the date of hepatic metastatectomy to the date of event (death from cancer or recurrence of cancer respectively). Survival curves were estimated by the Kaplan-Meier method. In case of no event patients were censored at the date of last follow-up. The log-rank test was used to evaluate differences between survival curves of different groups. The median concentration or proportion values of $CD8^+$ T-cells or $FoxP3^+$ T-cells were used to discriminate high vs low groups in both immunohistochemistry and flow cytometry. For multivariate analysis, the Cox proportional Hazard regression analysis was used with backward variable selection. Patients with missing values for the co-variates of interest were excluded from the statistical analysis. The associations between clinicopathologic parameters and the TIL subpopulations were examined using the χ^2 tests or the T-test as appropriate. The statistical analysis was performed using the SPSS© 21 software.

RESULTS

Patients and baseline clinicopathologic characteristics.

Median time to recurrence was 13.1 months and median survival was 50.9 months. Cancer recurrence occurred in 37/47 patients while 28/47 patients died from colorectal cancer metastasis. Baseline clinicopathologic characteristics can be seen in Table 1. The univariate hazard ratios for all the baseline clinicopathologic characteristics can be seen in Table 2. Only the lymph node status of the primary cancer was associated with colorectal cancer death in our cohort (HR 2.48, 95% CI 1.10-5.60, p=.024). The CRS did not predict colorectal cancer death in our cohort.

Table 1

	Baseline characteristics	N=47 (% or range)	Missing values
	Age (median)	63.8 (35.2-84.7)	
	Gender (male/female)	31 (66.0) / 16 (34.0)	
Clinical Risk Score	Primary cancer lymph node status positive	26 (56.5)	1
	Time from primary to CRCLM < 12 months	17 (36.2)	
	Largest metastatectomy tumor size >5cm	11 (23.4)	
	>1 metastatic lesion	30 (63.8)	
	CEA >200 ng/ml	1 (5.9)	30
	Recurrence	37 (78.7)	
	Cancer specific death [a]	28 (59.6)	

[a] All patients who died, died from colorectal cancer metastasis

Description of $CD8^+$ and $FoxP3^+$ TIL distribution.

The majority of TILs were located in the peri-tumoral areas (Figures 1 and 2). Median intra-tumoral $CD8^+$ and $FoxP3^+$ TIL concentrations were 58.6/mm^2 (range 8.8-795) and 26.4/mm^2 (range 0.44-255) respectively. Median peri-tumoral $CD8^+$ and $FoxP3^+$ TIL concentrations were 883/mm^2 (range 409-1,731) and 127/mm^2 (range 8.4-525) respectively. $CD8^+$ peri-tumoral TILs outnumbered intra-tumoral $CD8^+$ TILs by a factor of 15 while $FoxP3^+$ peri-tumoral TILs outnumbered intra-tumoral $FoxP3^+$ TILs by a factor of 5.

Prognostic value of intra-tumoral and peri-tumoral $CD8^+$ and $FoxP3^+$ TILs by immunohistochemistry

Median values were used for cutoffs. Table 2 shows the Hazard Ratios and 95% confidence intervals for the high versus low TIL populations in the intra-tumoral and peri-tumoral areas while Figure 3 shows the respected Kaplan-Meier curves. Intra-tumoral nor peri-tumoral areas $CD8^+$ and $FoxP3^+$ TIL concentrations did not, individually, predict colorectal cancer death. An *a priori* hypothesis was that the ratio of $CD8^+$ to $FoxP3^+$ TILs

would be a better prognosticator than the individual TIL sub-populations. Indeed, a high intra-tumoral $CD8^+/FoxP3^+$ TIL ratio was predictive of improved colorectal cancer survival (HR 0.43, 95% CI 0.19-0.95, p=.032). On the other hand, the peri-tumoral $CD8^+/FoxP3^+$ TIL ratio was not predictive of colorectal cancer survival. None of the TIL parameters were predictive of colorectal cancer recurrence, although a high concentration of intra-tumoral $CD8^+$ TILs (HR 0.51, 95% CI 0.25-1.02, p=.052) showed a trend towards less colorectal cancer recurrence.

Table 2 Univariate Cox proportional Hazard regression analysis of patients' survival

	Variables	HR	95% CI	p-value
Baseline clinicopathologic characteristics	Age (per decade increase)	0.99	0.71-1.38	.957
	Gender (male vs female)	0.78	0.35-1.64	.485
	Primary cancer lymph node status positive	**2.48**	**1.10-5.60**	**.024**
	Time from primary to CRCLM < 12 months	1.01	0.47-2.16	.977
	Largest metastatectomy tumor size >5cm	1.17	0.50-2.75	.730
	>1 metastatic lesion	1.07	0.49-2.31	.871
	CEA >200 ng/ml*	7.30	0.66-80.6	.164
	CRS (0-5)	1.24	0.87-1.77	.231
TIL parameters	CD8 peri-tumoral area	0.80	0.38-1.75	.571
	FoxP3 peri-tumoral area	1.08	0.50-2.29	.852
	CD8/FoxP3 peri-tumoral area	0.91	0.43-1.95	.813
	CD8 intra-tumoral area	0.58	0.27-1.27	.169
	FoxP3 intra-tumoral area	0.83	0.39-1.77	.622
	CD8/FoxP3 intra-tumoral area	**0.43**	**0.19-0.95**	**.032**

* Hazard Ratio for the CEA parameter represents 1 single case of CEA >200 ng/ml out of 17 patients with available baseline values.

Prognostic value of intra-tumoral TILs by flow cytometry.

Flow cytometry using freshly isolated TIL was used to validate our immunohistochemical findings. Flow cytometry was limited to tissue from the intra-tumoral compartment. Median values were used for cutoffs. While $CD8^+$ TILs were not predictive of colorectal cancer survival (HR 0.88, 95% CI 0.41-1.91, p=.753), high percentages of Tregs within CD45+ leukocytes were associated with worse colorectal cancer survival (HR 2.61, 95% CI 1.17-5.83, p=.016). In addition, high $CD8^+$/Treg TIL ratio was associated with improved colorectal cancer survival (HR 0.45, 95% CI 0.20-0.99, p=.044). Figure 3 shows the representative Kaplan-Meier curves. In contrast, TIL measurement by flow cytometry was not predictive of colorectal cancer recurrence.

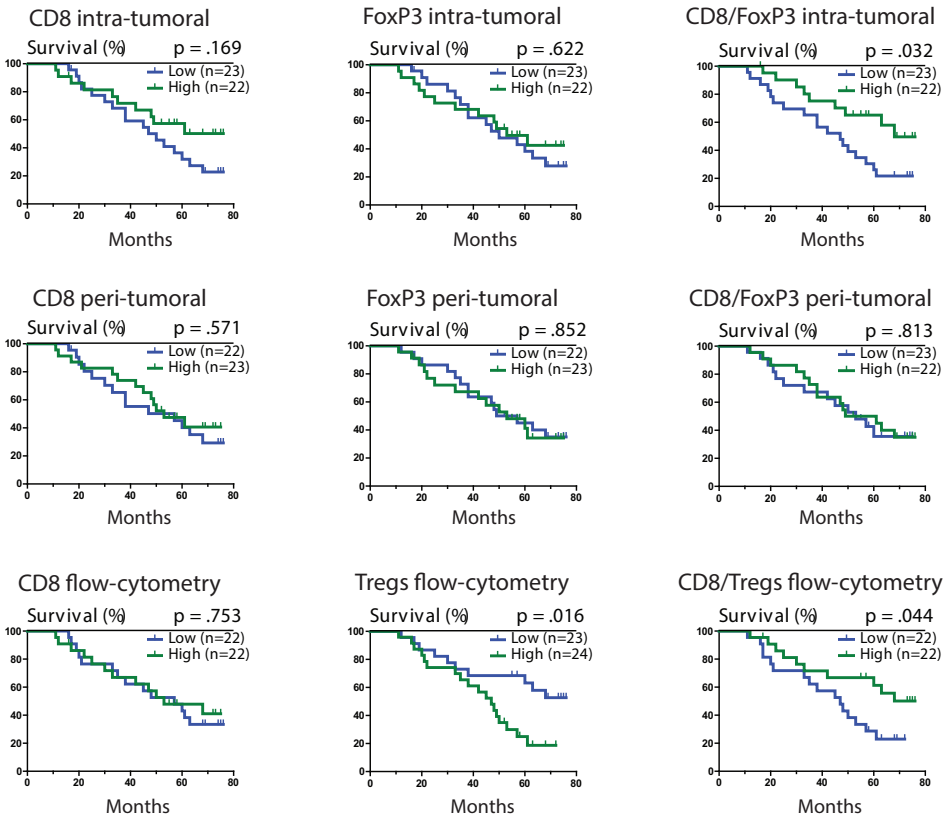


Figure 3. Kaplan Meier curves of potential TIL biomarkers. $CD8^+$ and $FoxP3^+$ cell concentrations were measured as numbers of cells/mm^2 tissue in immunohistochemistry and as proportions of $CD8^+$ or $CD4^+CD25^+FoxP3^+$ cells within $CD45^+$ leukocytes by flow cytometry. Median numbers or proportions were used as cutoff levels. Note that only the intra-tumoral $CD8^+/FoxP3^+$ ratios obtained by immunohistochemistry and flow cytometry and intra-tumoral Treg proportions as determined by flow cytometry show statistically significant differences in colorectal cancer survival times.

Comparison of immunohistochemical intra-tumoral $CD8^+/FoxP3^+$ TIL ratio with the $CD8^+$/Treg TIL ratio obtained by flow cytometry

There was no correlation between the intra-tumoral $CD8^+/FoxP3^+$ TIL ratio obtained by immunohistochemistry and the $CD8^+$/Treg TIL ratio obtained by flow cytometry (Spearman's Correlation Coefficient = .026, p = .871). Patients with high intra-tumoral $CD8^+/FoxP3^+$ TIL ratio determined by immunohistochemistry did not tend to also have high $CD8^+$/Treg TIL ratio as determined by flow cytometry (Table 3).

Table 3 Crosstabs analysis of high vs low intra-tumoral $CD8^+/FoxP3^+$ TIL ratio obtained by immunohistochemistry and high vs low $CD8^+$/Treg TIL ratio obtained by flow cytometry

		CD8+/Treg TIL ratio (Flow Cytometry)		
p=.346		Low	High	Total
Intra-tumoral CD8+/FoxP3+ TIL ratio (Immunohistochemistry)	Low	12	10	22
	High	8	12	20
	Total	20	22	42

Multivariate analysis

Multivariate analysis was performed by including all known clinicopathologic characteristics and the TIL ratios predictive of colorectal cancer survival (Table 4). Both the intra-tumoral $CD8^+/FoxP3^+$ TIL ratio obtained by immunohistochemistry and the $CD8^+$/Treg TIL ratio obtained by flow cytometry are independent predictors of colorectal cancer survival. From the clinicopathologic characteristics only lymph node status of the primary tumor was an independent predictor of colorectal cancer death.

Table 4a Multivariate Cox proportional Hazard regression analysis of intratumoral $CD8^+/FoxP3^+$ TIL ratio obtained by immunohistochemistry adjusted for clinicopathologic characteristics

Variables	HR	95% CI lower limit	95% CI upper limit	p-value
Primary cancer lymph node status	**3.80**	**1.12**	**2.24**	**.004**
Time to CRCLM < 12 months	0.68	0.28	1.65	.394
Largest tumor size >5cm	0.57	0.20	1.76	.341
>1 metastatic lesion	1.53	0.61	3.84	.361
Intra-tumoral $CD8^+/FoxP3^+$ TIL ratio	**0.31**	**0.12**	**0.79**	**.014**

Table 4b Multivariate Cox proportional Hazard regression analysis of $CD8^+$/Treg TIL ratio obtained by flow cytometry adjusted for clinicopathologic characteristics

Variables	HR	95% CI lower limit	95% CI upper limit	p-value
Primary cancer lymph node status	**2.90**	**1.14**	**7.40**	**.026**
Time to CRCLM < 12 months	1.06	0.44	2.56	.899
Largest tumor size >5cm	0.48	0.15	1.50	.206
>1 metastatic lesion	0.89	0.36	2.21	.800
$CD8^+$/Treg TIL ratio	**0.38**	**0.16**	**0.92**	**.031**

DISCUSSION

In our study, we show that the intra-tumoral ratio, but not the peri-tumoral ratio, of effector T-cells ($CD8^+$ cells) to immune inhibitory T-cells ($FoxP3^+$ T-cells), measured by im-

munohistochemistry, is a predictor of cancer survival in the setting of resected CRCLM. In contrast, intra-tumoral $CD8^+$ T-cells or $FoxP3^+$ T-cells, individually, could not predict cancer survival. In addition, we show that measuring the same ratio of effector T-cells ($CD8^+$ cells) to immune inhibitory T-cells (Tregs) by flow cytometry, using freshly isolated TILs, can also predict cancer survival following colorectal cancer liver metastasis.

This observation is consistent with the hypothesis that it is not the quantity of the immune infiltrate but the quality that matters the most [18]. Specifically, ratios of effector to regulatory immune cells may provide a more comprehensive and meaningful view of what occurs at the tumor microenvironment than numbers of individual types of effector or regulatory immune cells. In fact, a large meta-analysis of TIL phenotyping, including 33 studies and nearly 10,000 patients in various cancers, showed that lymphocyte ratios, and specifically the CD8/FoxP3 ratio, have more prognostic potential than individual lymphocytic subtype concentrations [19]. The same reason may also explain why high numbers of intra-tumoral Tregs measured by flow cytometry were associated with worse survival while intra-tumoral Tregs measured by immunohistochemistry were not. In contrast to immunohistochemistry, Tregs measured by flow cytometry are calculated as proportions of $CD45^+$ leukocytes, thus providing a ratio of regulatory to total immune cells.

The fact that we observed a prognostic significance only for the intra-tumoral and not for the peri-tumoral $CD8^+$/$FoxP3^+$ ratio also makes sense. While a lymphocytic infiltrate may be attracted to the margin of the tumor it does not mean, by default, that such a lymphocytic infiltrates lead to tumor disruption. We found much higher concentrations of $CD8^+$ T cells and $Foxp3^+$ Tregs around the tumors than in the tumors. Apparently, CRCLM prevent the migration of these immune cells into the tumor itself, which may be due to chemokine to chemokine receptor mismatch, aberrant microvasculature, cytokine production and immunosuppression itself [20]. On the other hand, intra-tumoral lymphocytes that are in direct contact with tumor cells are more likely to have a significant anti-cancer role.

Four prior studies have attempted to examine the prognostic role of TILs in CRCLM. The study by Halama et.al., [12] examined full slides taken from 101 patients with CRCLM and found that high TIL "density" was associated with improved survival. However, the prognostic significance of TIL for CRCLM resection was examined in only 33 of the patients since the rest of the patients (n=68) had unrespectable disease and were treated with neo-adjuvant chemotherapy at the time of presentation. TIL infiltration was measured in an area within 500 μm from the tumor edge and included both the peri-tumoral and the intra-tumoral areas as defined by our study. Total immune infiltrate was defined

as a combination of CD3, CD8 and granzyme B staining. Thus, since Halama et.al. did not differentiate intra-tumoral from peri-tumoral lymphocytic infiltration in CRCLM it is difficult to compare their results with ours. A study by Katz et.al., [11] used three 0.6mm TMA cores per patient, taken from 188 patients with CRCLM. They showed that a high CD8/FoxP3 ratio (or a low FoxP3/CD8 ratio as defined in their study) predicts improved survival following CRCLM resection, a finding which is in agreement with our study. The fact that they used only three small TMA cores means they examined a much smaller area of tumor than in our study but reached a similar conclusion nonetheless. The TMA cores from the Katz et.al. study should correspond to the intra-tumoral areas in our study, likely explaining the similarity in our results [11]. Katz et.al., however, did not examine the peri-tumoral areas, given the inability of TMAs to reliably capture the histologic complexity of the cancer microenvironment. A study by Lee et.al., [21] examined TMAs consisting of 5mm cores taken from 79 patients with synchronous only CRCLM. TILs in the liver metastasis was compared to TILs in the corresponding primary tumors. While in univariate analysis a higher $FoxP3^+$ density at the metastatic site corresponded to better outcome, in multivariate analysis only $CD45RO^+$ cell density at the primary site was prognostic of survival. Our study did not examine the relative role of TIL infiltration of the corresponding primary sites thus is difficult to compare our findings with the study by Lee et.al. More importantly however, it is unclear where the TMA cores were taken from. The relatively large 5mm cores may have included both peri-tumoral and intra-tumoral infiltrates. In addition, Lee at.al., did not report on the CD8/FoxP3 ratio. These differences may explain the different results between the study of Lee et.al on the one hand and our study and that of Katz et.al. on the other hand. Finally, a more recent study by Nakagawa et.al., [10] examined full slides from 162 patients with CRCLM. Both intra-tumoral and peri-tumoral areas were examined according to the authors. High peri-tumoral FoxP3 density was found to be associated with improved survival, while peri-tumoral CD8 and intra-tumoral CD8 and FoxP3 densities did not predict survival individually. Ratios of CD8 to FoxP3 densities were not examined. In addition, no definition of what constituted peri-tumoral or intra-tumoral areas was given.

It is clear from the above studies that significant heterogeneity is study design and terminology exists. In fact, differences in methodology, rather than differences in biology, have been hypothesized before to account in differences amongst study observations [19]. In the case of CRCLM, only two prior studies [10, 12] have used full slides, allowing for comparison of intra-tumoral and peri-tumoral TIL populations and none of these studies commented on the possible significance of the CD8/FoxP3 ratio, which we show to be the strongest prognostic marker. On the other hand, the study by Katz et.al., which found the CD8/FoxP3 ratio to be prognostic of survival, concordant to our results, did

not examine the peri-tumoral infiltrate [11]. While we validate the findings of Katz et.al., we show, in addition, that it is indeed the intra-tumoral infiltrate that matters the most.

A novelty of our study is the validation of our immunohistochemical observations by using flow cytometry of TILs isolated from fresh tissues, collected prospectively, from these same patients. We show that a high flow cytometric $CD8^+$/Treg TIL ratio is also associated with improved colorectal cancer survival following CRCLM resection. It was surprising however, at first sight, that the results obtained by immunohistochemistry did not correlate with the results obtained, on a patient by patient basis, by flow cytometry. The reasons for this discrepancy may be several. Firstly, immunohistochemistry measures absolute numbers of cells over a specific area of tissue, while flow cytometry measures proportions of cells within the total isolated cells (in this case intra-tumoral $CD45^+$ leukocytes). Secondly, by simultaneous determination of several molecules on the same cells, flow cytometry is able to define cell subpopulations much more specifically than immunohistochemistry. Tregs were defined as $CD45^+$ $CD8^+$ $CD4^+$ $CD25^+$ $FoxP3^+$ by flow cytometry while that level of specificity is impossible by immunohistochemistry, where traditionally FoxP3 staining alone is used to define Treg cells. Furthermore, FoxP3 in humans, in contrast to mice, can also be expressed by activated helper T-cells [22, 23]. In addition, activation-induced FoxP3 in human T-effector cells does not suppress proliferation or cytokine production [23]. Therefore, flow cytometric enumeration of Treg cells is more accurate and may account for the differences observed in our study between flow cytometry and immunohistochemistry.

While we show that the intra-tumoral $CD8^+$/$FoxP3^+$ ratio is prognostic of colorectal cancer survival, no association with colorectal cancer recurrence was seen. It is likely however, that overall survival reflects the biologic behavior of colorectal cancer much better than recurrence, and it is thus a better endpoint to study in the setting of resected CRCLM. While cancer recurrence is undoubtedly a surrogate endpoint of cancer survival, many patients with recurrence can still enjoy long term survival, or cure, following additional interventions and palliative treatments. In that case, cancer recurrence does not represent aggressive biologic behavior of cancer, but rather remaining metastatic lesions too small to be clinically detected at the time of the initial resection.

Our study has several strengths. We used full slides to examine the complex histologic distribution of TIL in the tumor microenvironment and we carefully defined the intra-tumoral and peri-tumoral infiltrates. We also looked at the spatial distribution of effector to regulatory cells. Finally, we used a second technique, flow cytometry, to better define regulatory T-cells in tumors and, at least partially, validate the immunohistochemistry findings. Our study, however, has also limitations. The main limitation relates to the

sample size of our study (n=47). While our sample size is comparable to the sample size of other similar studies using full slides, it is undoubtedly small. The reason for this was the need for prospective collection of freshly resected tumor material for flow cytometry, which limits the number of patients to more recent resections. At the same time, the need for adequate time to follow up excludes patients resected too recently for inclusion. . However, despite the small sample size, we were able to statistically confirm our *a priori* generated hypothesis, that a high intra-tumoral $CD8^+$/$FoxP3^+$ ratio would be protective in patients following resection for CRCLM. Therefore, our study confirms the conclusion of Katz et. al. [11], that the intra-tumoral CD8/FoxP3 ratio is an independent predictor of survival after CRCLM resection and adds the observation that peri-tumoral $CD8^+$ and $Foxp3^+$ T cells are not associated with survival.

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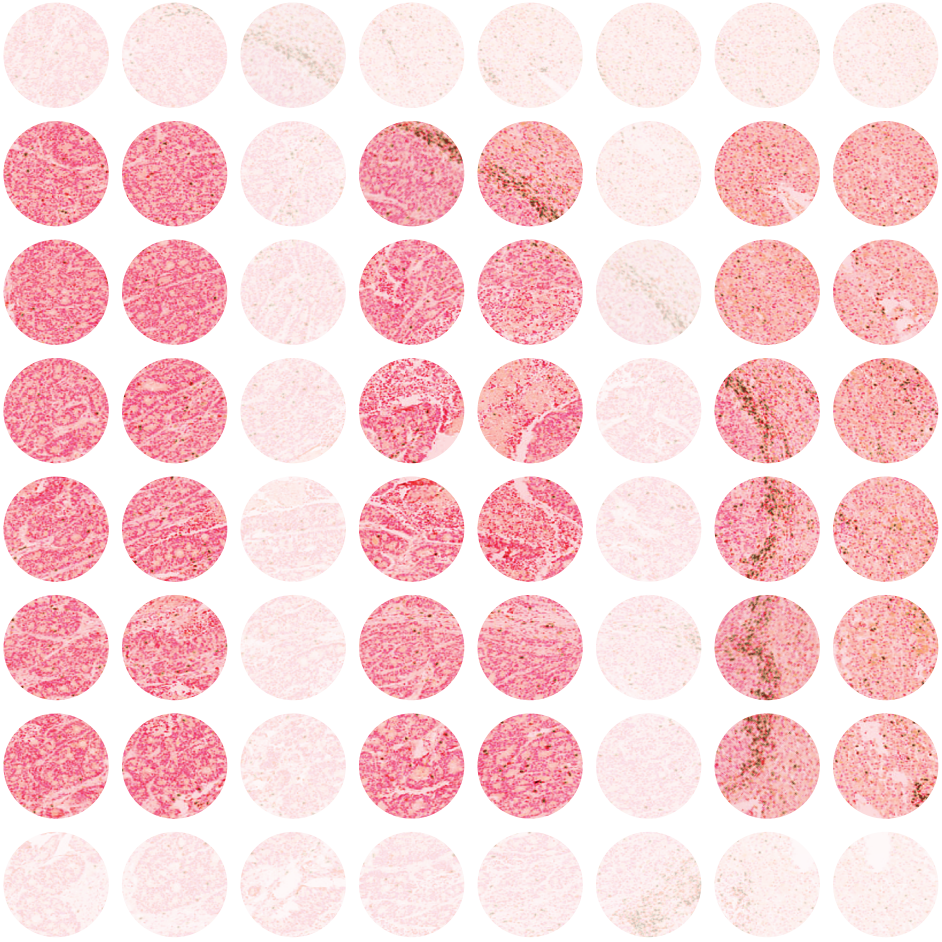
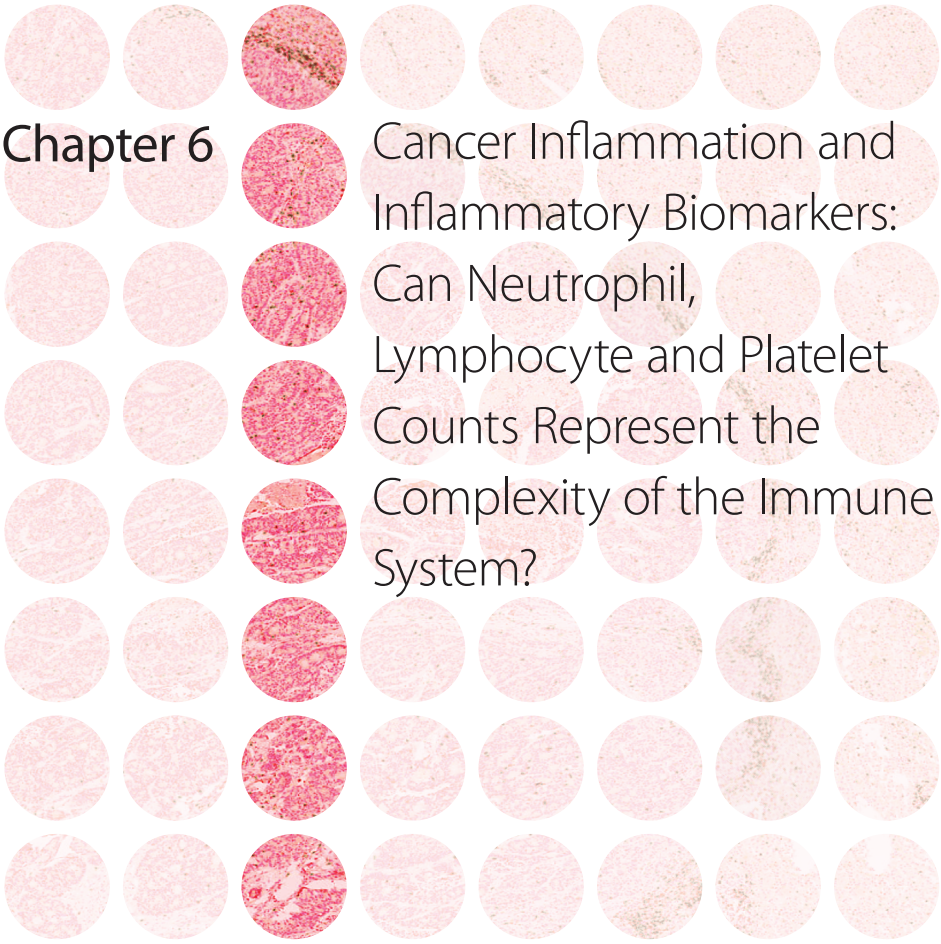


PART III

CIRCULATING IMMUNE BIOMARKERS



Chapter 6

Cancer Inflammation and Inflammatory Biomarkers: Can Neutrophil, Lymphocyte and Platelet Counts Represent the Complexity of the Immune System?

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INVITED COMMENTARY

Despite the fact that the link between cancer and inflammation has been first suggested by Virchow in the 19th century it has only been in the last 15 years that our understanding of this link has reached the point where therapeutic interventions are possible [1-3]. Inflammation is known to be both a cause and a consequence of cancer. For example chronic inflammation due to infectious diseases is believed to be responsible for more than 15% of known cancers to date [4] while inflammatory mediators are known to directly promote malignant transformation in experimental models [5]. On the other hand, once cancer progresses it leads to a chronic inflammatory-like state which, through altered aminoacid metabolism amongst other mechanisms, causes cachexia, the main cause of death in cancer patients [6].

Given that multiple immune and inflammatory markers are part of routine laboratory testing their use as prognostic and predictive biomarkers has been extensively examined. For example a low absolute lymphocyte count (ALC), generally less than 1.5 or 1.2 x 10^9/L, is prognostic of poor survival in the setting of multiple cancers such as non-Hodgkin's lymphoma, chronic lymphocytic leukemia, acute leukemia, head and neck cancer, cancers of the ovary, breast, colon, pancreas and lung as well as sarcomas [7-12]. Moreover, low ALC is predictive of poor response to chemotherapy in colorectal, lung and breast cancer [10]. In addition to single markers, such as ALC, prognostic scores based on combining various inflammatory markers have been developed. Examples include the modified Glasgow prognostic score (C-reactive protein and albumin) [13], the prognostic index (C-reactive protein and white cell count) [14], the neutrophil to lymphocyte ratio (NLR) and the platelet to lymphocyte ratio (PLR). From these biomarkers the NLR, which has been examined in over 60 studies, has been shown to be prognostic of outcomes in multiple cancers [15]. For example a recent meta-analysis in colorectal cancer patients, which included 16 studies, showed that an elevated NLR is indeed associated with poor survival [16].

Efforts in developing such inflammatory biomarkers have also been made in hepatocellular carcinoma (HCC) either following resection, trans-arterial chemoembolization or liver transplantation. Halazun et. al. showed that amongst 150 patients undergoing liver transplantation for HCC those with an NLR above 5 had a significantly lower overall survival (5-year survival, 28% vs. 64%, $P = 0.001$) and NLR was the only significant factor in predicting disease free survival in multivariate analysis [17]. Six additional studies (4 of them very recent) have now supported the role of the elevated NLR as a powerful prognostic marker of HCC recurrence following liver transplantation, although they used different cutoffs ranging from ≥ 3 to ≥ 5 [18-23].

In the study by Lai et. al., in this issue of Transplant International [24], NLR as well as PLR was examined as a prognostic biomarker in 181 patients undergoing liver transplantation for HCC. Indeed an elevated NLR (≥ 5.4), in agreement with older and current literature, was again prognostic of poor survival in these patients (5-year survival rate of 48.2% vs. 64.5%). The novelty of the current study resides in the use of the NLR as a predictor of drop-out from the waiting list. The last NLR measurement, performed just before transplantation or drop-out, but not the initial value at the time of listing, nor the slope, was the best predictor of drop-out of all parameters examined (43% for NLR ≥ 5.4 vs 21% for NLR < 5.4). While this study introduces an inflammatory biomarker, such as the NLR, as a possible predictor of drop-out its clinical utility at the current time is limited by the fact that the information, if validated, is available to the clinician too late. Clearly, examination of NLR as a predictor of drop-out should be examined in prospective studies in which regular measurements during the waiting list period are performed, since there is a real need for the identification of such biomarkers.

A limitation in the use of peripheral blood inflammatory markers for the prognostication of HCC patients undergoing transplantation is the fact that inflammation due to hepatitis infection, hepatic cirrhosis, or the use of immunosuppressive drugs post transplantation will inadvertently have an impact on these markers irrespective of tumor biology. As a result, the composition of the inflammatory milieu at the site of the tumor microenvironment, while not easily accessible, may be more informative of tumor biology than peripheral blood markers. The prognostic ability of the intratumoral immune infiltrate has now been shown in all major cancers and is probably best exemplified in colorectal cancer where the immune infiltrate at the primary tumor site of more than 400 patients was prognostically superior to clinical parameters including the TNM stage [25]. This means that information from the immune infiltrate can provide prognostic information superior to the size of the cancer (T) or the lymph nodes metastasis status (N) [26]. In the liver transplantation field Unitt et. al. showed that reduced lymphocytic infiltration in HCC tumors as well as a high intra-tumoral CD4 to CD8 ratio, were independent prognostic factors of poor outcome, consistent with the hypothesis that a reduced number of cytotoxic CD8+ effector T lymphocytes at the tumor site is a sign of poor immunological reactivity to the malignant cells [27].

Furthermore, it is now clear that the inflammatory reaction associated with cancer is a largely ineffective anti-tumor response, and that the local tumor environment is often infiltrated by immunosuppressive as well as tumor growth-promoting cells [28, 29]. Anti-tumor effector immune-type cells include cytotoxic CD8+ T cells, NK cells, NKT cells, Th1 helper cells and M1 macrophages, which secrete and are supported by cytokines such as IL-2, TNF-α and IFN-γ. Among immunosuppressive and tumor supporting cells,

which are recruited into tumors, are the T regulatory cells, myeloid derived suppressor cells, Th2 helper cells and tumor associated macrophages. These immunosuppressive cells secrete and are supported by cytokines such as IL-10 and TGF-β and VEGF, and their presence in tumor tissues of HCC patients is well documented [30-33]. Interestingly, although they can exert both tumor-promoting and tumor-killing functions, intratumoral neutrophils are a poor prognostic factor in HCC [34]. A further complicating issue is the expression by both immune cells and cancer cells of immune inhibitory ligands and other immunosuppressive molecules such as PD-L1, B7-H3, B7-H4, Gal-9, indoleamine 2, 3-dioxygenase (IDO), all of which inhibit antitumor immune responses and are currently the target of new cancer drugs [35].

Clearly routine laboratory tests, such as ALC, C-reactive protein and composites such as the NLR or other inflammatory indices, cannot capture the complexity of cancer inflammation and cancer immune responses or accurately represent tumor biology. In addition, knowledge of the immune interactions at the tumor microenvironment can provide clinicians with targets for treatment in an era where personalized medicine is the ultimate goal. This complexity however makes even the more remarkable the fact that such crude routine blood tests, such as the NLR, can demonstrate, in at least 8 studies now [17-24], such powerful prognostic ability in liver transplantation for HCC. While we are waiting for tumor immunologists to unravel all the secrets of the immune system there may just be time for patients to benefit from the cheap and easily accessible information available from a routine blood draw.

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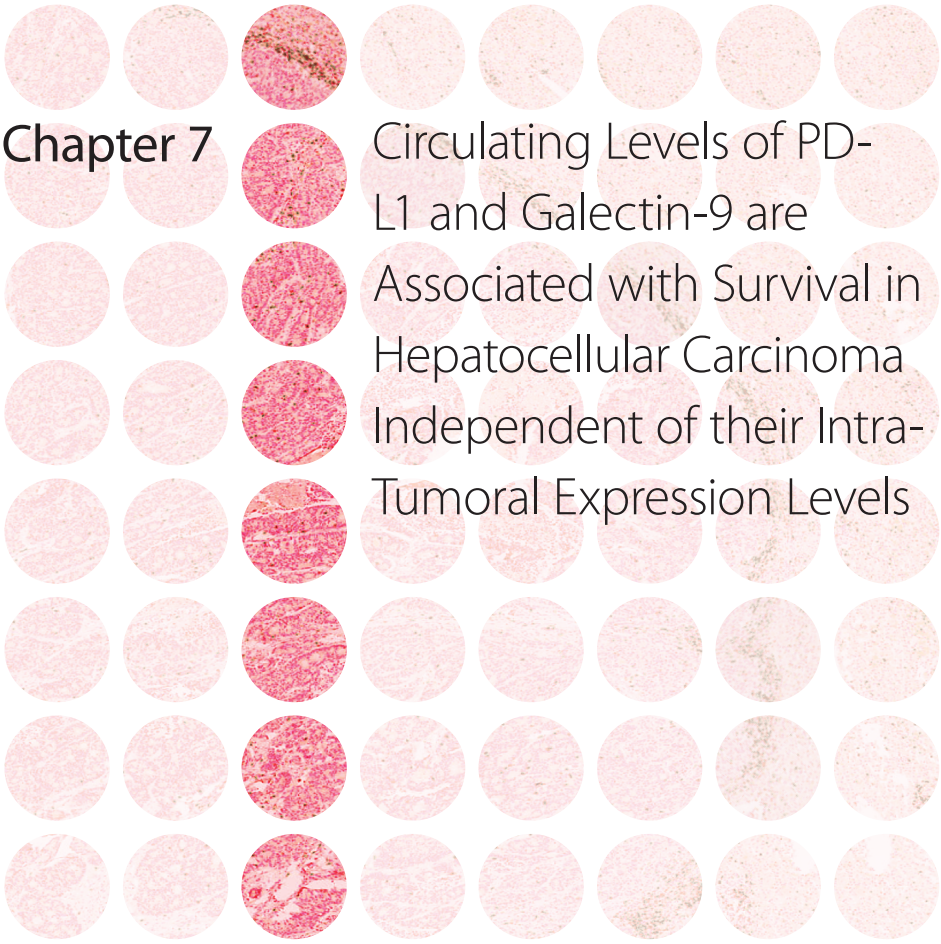

Chapter 7

Circulating Levels of PD-L1 and Galectin-9 are Associated with Survival in Hepatocellular Carcinoma Independent of their Intra-Tumoral Expression Levels

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ABSTRACT

Background: Tumor expression of co-inhibitory ligands, such as PD-L1 and Galectin-9, has prognostic value in Hepatocellular Carcinoma (HCC) and other types of cancer. In addition, intra-tumoral PD-L1 expression seems predictive for the therapeutic effect of anti-PD-1 antibodies, in some types of cancer. Determination of tumor tissue expression of these molecules requires tumor biopsy or surgical intervention. To facilitate prognostication, less invasive prognostic biomarkers, such as circulating PD-L1 or Galectin-9, would be preferable. Therefore, the aims of the study were to access the prognostic significance of circulating levels of PD-L1 and Galectin-9 in HCC patients and to compare the prognostic significance to the intra-tumoral expression of these molecules.

Methods: Archived tissues and stored peripheral blood samples from 81 patients who underwent HCC resection or liver transplantation, with curative intent, were used. Immunohistochemistry was performed to determine intra-tumoral expression of PD-L1 and Galectin-9, while ELISA was used to quantify their circulating levels.

Results: High circulating PD-L1 (HR 0.12, 95%CI 0.16-0.86, p=.011) and high circulating Galectin-9 (HR 0.11, 95%CI 0.15-0.85, p=.010) levels were both associated with improved HCC-specific survival and recurrence. Surprisingly, there was no correlation between circulating levels of PD-L1 and Galectin-9 and their intra-tumoral expression levels. In fact, circulating levels of PD-L1 and Galectin-9 were predictive of HCC-specific survival independently of intra-tumoral levels and baseline clinicopathologic characteristics. Combined analysis of circulating levels and intra-tumoral expression of PD-L1 (HR 0.33, 95%CI 0.16-0.68, p=.002) and Galectin-9 (HR 0.27, 95%CI 0.13-0.57, p=.001) resulted in more confident prediction of survival.

Conclusion: Circulating PD-L1 and Galectin-9 levels prognostically differentiate resected HCC patients, independently of their intra-tumoral expression. Combined circulating and intra-tumoral expression levels of PD-L1 or Galectin-9 are associated with more confident prognostic immune biomarker profiles.

INTRODUCTION

Worldwide over half a million people die from HCC every year (1, 2). Only 20% of patients with HCC are diagnosed early enough to be candidates for curative treatments such as resection, local ablation or liver transplantation (3). Once advanced disease is diagnosed, HCC is incurable and overall survival can be only modestly extended with sorafenib (4, 5).

Developments in our understanding of tumor immunology (6) have brought forth new therapeutic strategies against cancer (7). Currently, the most successful immunotherapeutic strategies are those that are designed to overcome immune resistance mechanisms by using antibodies that abrogate co-inhibitory receptor-ligand interactions, the so-called negative immune checkpoint inhibitors (8). Recent approval of anti-CTLA-4 and anti-PD-1 antibodies for the treatment of advanced melanoma, non-small cell lung cancer, renal cell carcinoma and bladder cancer (9-14) has made it clear that immunotherapy is the new wave of anti-cancer treatments. Immunotherapy clinical trials are now ongoing in many cancers, including HCC, and it is likely that these therapies will be approved in the future in other cancer types as well. In view of the high costs and occasional severe toxicity of these novel therapies, immune specific biomarkers that can predict which patients will benefit are urgently needed. Two such recent promising biomarkers in HCC are Programmed Death Ligand-1 (PD-L1) and Galectin-9 (Gal-9).

PD-L1 is a ligand that binds PD-1, a co-inhibitory receptor expressed on activated T cells. Binding of the ligand PD-L1 to its receptor PD-1 transduces a negative signal into T-cells inhibiting their activation (15). In HCC the PD-L1/PD-1 interaction impairs effector T-cell function and *in vitro* disruption of this interaction restores the function of tumor-derived effector T-cells (16, 17). PD-L1 is known to be expressed by HCC cells (16-23). We have previously shown that tumor PD-L1 protein expression is a promising prognostic biomarker in HCC (24). In addition, tumor PD-L1 protein expression has shown promise as a predictive biomarker to identify cancer patients that respond to anti-PD1 immunotherapy (25, 26).

Gal-9 is a glycan-binding protein and an important modulator of T-cell function (27). Gal-9 causes T-cell inhibition and apoptosis through its binding to the co-inhibitory receptor TIM-3, and blockade of the interaction between Gal-9 and TIM-3 reinvigorates *ex vivo* responses of T-cells of HCC and melanoma patients to tumor antigens (28, 29). Humanized antagonistic antibodies against TIM-3 are currently in preclinical development (30). In addition, binding of Gal-9 to CD44 enhances the differentiation of immunosuppressive T regulatory cells (31). A direct anti-metastatic role for Gal-9 has also been described (32,

33). We and others have demonstrated that Gal-9 is also expressed by HCC cells and that Gal-9 protein expression is a potential prognostic biomarker in HCC (24, 29, 34).

However, in addition to cell-bound expression, soluble forms of PD-L1 and Gal-9 exist in the circulation. These circulating forms of PD-L1 and Gal-9 have been poorly studied in cancer patients. For example, while circulating levels of PD-L1 have been examined in renal-cell cancer (35), diffuse large B-cell lymphoma (36), lung cancer (37) and gastric cancer (38), no study has examined circulating levels of PD-L1 in HCC patients. Elevated levels of circulating Gal-9 have been observed in metastatic colon cancer (39) and in benign inflammatory liver diseases such as chronic hepatitis-C and active chronic hepatitis-B infection (40-42). No study has investigated circulating Gal-9 levels in relation to cancer survival in any cancer.

One may hypothesize that circulating forms of PD-L1 or Gal-9 would correlate with their tumor tissue expression status, since release from tumor cells, or from the tumor microenvironment, may be the source of these molecules in the circulation. In that case, circulating PD-L1 and Gal-9 may act as preferred biomarkers given the accessibility of peripheral blood when compared to tumor tissue. On the other hand, these physiologic molecules may have functions in the circulation that are independent of the immune interactions ongoing in the tumor microenvironment. Thus, the potential for the circulating levels of PD-L1 and Gal-9 to act as independent prognostic biomarkers, or independent predictors of immunotherapy treatment efficacy, exists. No study has correlated tissue protein expression status of these co-inhibitory ligands with their circulating levels. Thus, the aims of our study were to examine how circulating levels of PD-L1 and Gal-9 compare to tissue expression of these molecules and whether circulating levels have the potential to replace tissue expression, or add to tissue expression, as potential immune biomarkers in HCC.

PATIENTS AND METHODS

Patient population and tissue samples

Archival blood samples (59 serum samples and 22 plasma samples) and formalin fixed paraffin-embedded tissue samples from 81 patients who underwent hepatic resection or liver transplantation for HCC at Erasmus MC-University Medical Center between January 2007 and March 2013, were used for this study. All patients had undergone procedures with curative intent. Medical ethical approval was obtained from the Medical Ethical Committee of Erasmus MC. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Enzyme-linked immunosorbent assay

Sera or plasma samples were drawn no earlier than 4 months before the operation and were stored in -80°C. Soluble PD-L1 ELISA was performed as previously described (35) in the laboratory of Dr. Haidong Dong (Mayo Clinic, MN., US). Soluble Gal-9 was performed using an ELISA kit according to the manufacturers protocol (Uscn Life Science Inco, Wuhan China). All samples were tested in duplicate and mean values were used for analysis.

Tissue microarray (TMA) construction

TMAs were constructed as previously described (24). In brief, three 0.6 mm cores were taken from the tumorous areas and two 0.6mm cores were taken from the surrounding tumor free liver (TFL) tissue of each tissue block. The tumorous areas with vital tissue were marked by an experienced pathologist (KB) using archived H&E glass slides. The TMAs were made using an automated tissue-arrayer ATA-27 (Beecher Instruments, Silver Springs MD, USA).

Immunohistochemistry and scoring

Immunohistochemistry was performed as previously described (24). In brief 4 µm thick sections were mounted on Superfrost Plus™ slides. The sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 15 minutes. Antigen retrieval was performed in a microwave for 10 minutes using the appropriate antigen retrieval buffer. After serum block, primary antibodies were applied at 4°C overnight. The primary antibodies were PD-L1 clone 405.9A11 (43) (kindly provided by Dr. Gordon J. Freeman, Dana-Farber Cancer Institute, Boston, MA, USA), and Gal-9 goat polyclonal (41) (R&D systems). HRP-conjugated anti-mouse or anti-goat IgG polymer secondary antibody (Envision™, DAKO) was then applied for 1 hour, followed by diaminobenzadine (DAB) as the chromogen detection method. The slides were stained with haematoxylin followed by dehydration. Negative controls consisted of omission of the primary antibody and appropriate positive control tissues included in the TMAs were used to evaluate specificity of all antibodies. Scoring was performed by 2 independent investigators (KS and HS) blinded to clinical outcome and differences resolved by mutual agreement. Only cytoplasmatic staining was observed for both antibodies. Intensity of tumor cell and hepatocyte staining was scored in a scale from zero to three. Intra-core heterogeneity of staining intensity of tumor cells or hepatocytes was rarely observed, thus percentages of positive tumor cells were not analyzed. Average values of the scores of the different cores were used for analysis.

Statistical analysis

All analyses were performed in duplicate. Survival curves were estimated by the Kaplan-Meier method. Survival was calculated from the date of surgery to the date of event

(recurrence or death), or date of last follow up. The patients who died from causes other than HCC were censored at their time of death. The log-rank test was used to assess differences between survival curves of different groups, while for biomarkers with three linearly associated levels the linear trend for factor levels was used. Optimal high vs low values were established by examining a grid of cutoffs and choosing the cutoff with the lowest -2 log likelihood. For multivariate analysis, the Cox proportional Hazard regression analysis was used. The associations between clinicopathologic parameters with immune biomarkers, as well as the co-relationship of the immune biomarkers with each other were examined using the χ^2 tests or the T-test as appropriate. Sensitivity analysis was performed in relation to sample source (serums versus plasma) and type of surgery (resection versus liver transplantation). The statistical analysis was performed using the SPSS© 21 software.

RESULTS

Patients and clinicopathologic characteristics

Circulating PD-1 and Gal-9 levels were study in 81 HCC patients who underwent hepatic resection or liver transplantation. Median time to cancer recurrence was 29.7 months and median survival was 34.2 months. Baseline clinicopathologic characteristics can be found in Table 1. In univariate analysis, tumor size > 3cm (HR 3.0, 95%CI 1.1-8.2, p=.032) predicted HCC-specific mortality and a pre-operative AFP level > 100 μgl^{-1} (HR 2.1, 95%CI 0.9-5.3, p=.092) showed a trend toward predicting HCC-specific mortality. Number of lesions, tumor differentiation and vascular invasion did not predict HCC-specific mortality in our cohort. Size > 3cm also predicted HCC recurrence (HR 2.5, 95%CI 1.2-5.3, p=.012).

Association of circulating PD-L1 and Gal-9 with recurrence and survival

Median circulating PD-L1 concentration was 383 pg/ml (IQR 206-774 pg/ml), and median circulating Gal-9 concentration was 21 pg/ml (IQR 3-44 pg/ml). With a cutoff of 700 pg/ml, high circulating PD-L1 was associated with improved HCC-specific survival (HR 0.12, 95%CI 0.16-0.86, p=.011), and with a cutoff of 42 pg/ml high circulating Gal-9 was also associated with improved HCC-specific survival (HR 0.11, 95%CI 0.15-0.85, p=.010). The respected Kaplan-Meier curves are shown in Figures 1A and 1B. Using the same cutoff's, similar significant relationships between circulating PD-L1 and Gal-9 and HCC recurrence were found (Figures 1C and 1D). There was no correlation between circulating PD-L1 and Gal-9 levels (R^2=.002, p=.68). In multivariate analysis, together with clinicopathologic characteristics, circulating Gal-9 (p=.022) could independently predict HCC-specific survival, while circulating PD-L1 (p=.077) and AFP > 100 μgl^{-1} (p=.060) showed a strong trend to association with HCC-specific survival.

Table 1

Baseline characteristics		N or median (% or range)
Age		60 (23-79)
Gender (male/female)		58 (71.6) / 23 (28.4)
Type of Surgery	Resection	58 (71.6)
	Liver Transplantation	23 (28.4)
AFP before resection		7.5ug/l (1-15,000)
Cirrhosis		44 (54.3)
Viral hepatitis	Hepatitis-B [a]	17 (21.0)
	Hepatitis-C [b]	10 (12.3)
Tumor size (cm)		3.6 (1-25)
Number of lesions	Single	59 (72.8)
	Multiple	22 (27.2)
Vascular invasion		46 (56.8)
Tumor differentiation	Well	26 (32.1)
	Moderate	45 (55.6)
	Poor	10 (12.3)
HCC recurrence		36 (44.4)
HCC specific death		21 (25.9)

[a] HBsAg(+) and/or anti-HBc positive

[b] Anti-HCV positive

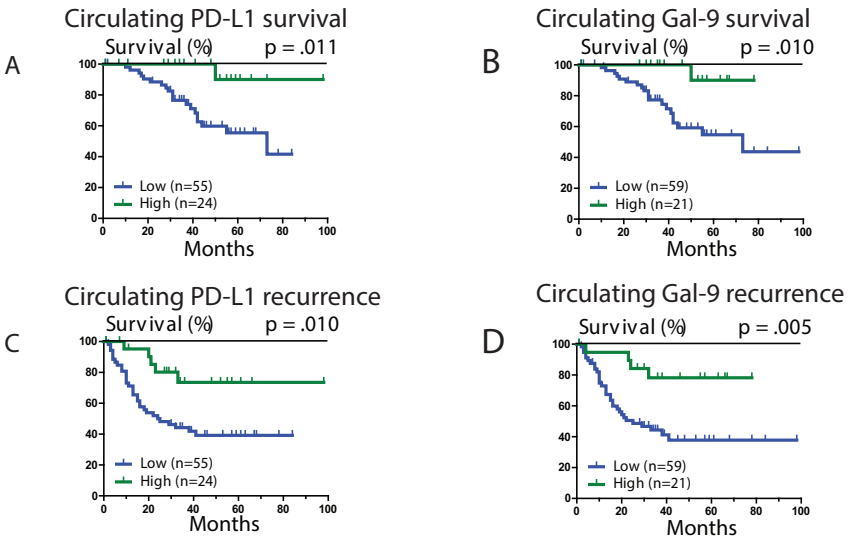

Figure 1. Kaplan-Meier graphs of circulating PD-L1 and Galectin-9. (A) HCC-specific mortality in HCC patients with high or low circulating PD-L1 concentrations. (B) HCC-specific mortality in HCC patients with high or low circulating Gal-9 concentrations. (C) Recurrence-free survival in HCC patients with high or low circulating PD-L1 concentrations. (D) Recurrence-free survival in HCC patients with high or low circulating Gal-9 concentrations.

Sensitivity analysis

In the majority of patients PD-L1 and Gal-9 were measured in sera but from 22 patients only plasma samples were available. When the patients with available plasma samples were excluded from the analysis the Kaplan-Meier survival curves, for the remaining 59 patients, are similar to Figures 1A and 1B (see Supplementary Figures 1A and 1B for comparison). In addition, the Kaplan-Meier survival curves of the 22 patients with plasma samples follow the same general direction as the patients with serum samples (Supplementary Figures 1C and 1D). Similarly, when the cohort is split between patients who underwent hepatic tumor resection versus liver transplantation, the Kaplan-Meier survival curves of circulating PD-L1 and circulating Gal-9 levels look similar and follow the same direction (Supplementary Figures 2A-2D). Thus, our results do not depend on the use of serum versus plasma samples or the type of surgery performed.

Association of intra-tumoral PD-L1 and Gal-9 with recurrence and survival

Examples of PD-L1 and Gal-9 stains of tumor tissues were shown in our previous paper on expression of these co-inhibitory ligands in HCC tumors (24). In this current cohort, PD-L1 expression in tumor cells was seen in 78% of cases for PD-L1 and 84% for Gal-9. Patients with any evaluable PD-L1 or Gal-9 staining on their tumor cells were considered to have high expression while patients with complete absence of staining were considered to have low expression. Patients with high intra-tumoral PD-L1 (HR 0.41, 95%CI 0.16-1.04, p=.051) and Gal-9 (HR 0.26, 95%CI 0.10-0.68, p=.003) had an improved HCC-specific survival (Figures 2A and 2B). The respective relationships of intra-tumoral PD-L1 and Gal-9 with HCC recurrence can be seen in Figures 2C and 2D. In multivariate analysis, intra-tumoral Gal-9 (p=.004), intra-tumoral PD-L1 (p=.035) and AFP > 100 μgl^{-1} (p=.004) were independent predictors of HCC-specific survival.

Circulating versus intra-tumoral and tumor-free liver PD-L1 and Gal-9

Next we examined if circulating levels of PD-L1 and Gal-9 reflected intra-tumoral expression. There was no correlation between intra-tumoral PD-L1 and circulating PD-L1 (R^2=.03, p=.130) or between intra-tumoral Gal-9 and circulating Gal-9 (R^2=.01, p=.349). In multivariate analysis, both intra-tumoral Gal-9 (p=.010) and circulating Gal-9 (p=.029) independently predicted HCC-specific survival, together with AFP > 100 μgl^{-1} (p=.016). In the case of PD-L1 , both intra-tumoral PD-L1 (p=.056) and circulating PD-L1 (p=.067) showed a strong trend towards independently predicting HCC-specific survival, together with AFP > 100 μgl^{-1} (p=.039). In addition, there was no correlation between circulating levels and expression levels of PD-L1 or Gal-9 on hepatocytes in TFL.

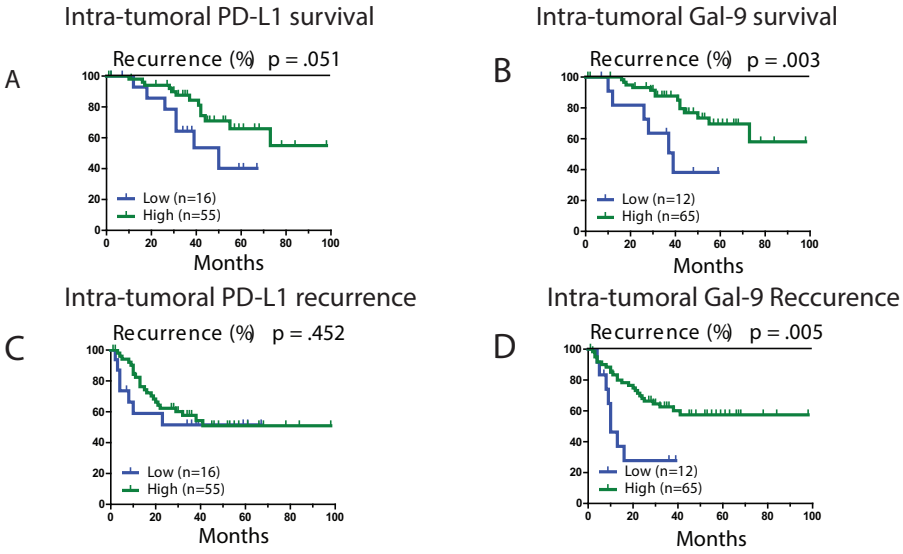

Figure 2. Kaplan-Meier graphs of intra-tumoral PD-L1 and Galectin-9. (A) HCC-specific mortality in patients with high or low intra-tumoral PD-L1 staining. (B) HCC-specific mortality in patients with high or low intra-tumoral Gal-9 staining. (C) Recurrence-free survival in patients with high or low intra-tumoral PD-L1 staining. (D) Recurrence-free survival in patients with high or low intra-tumoral Gal-9 staining.

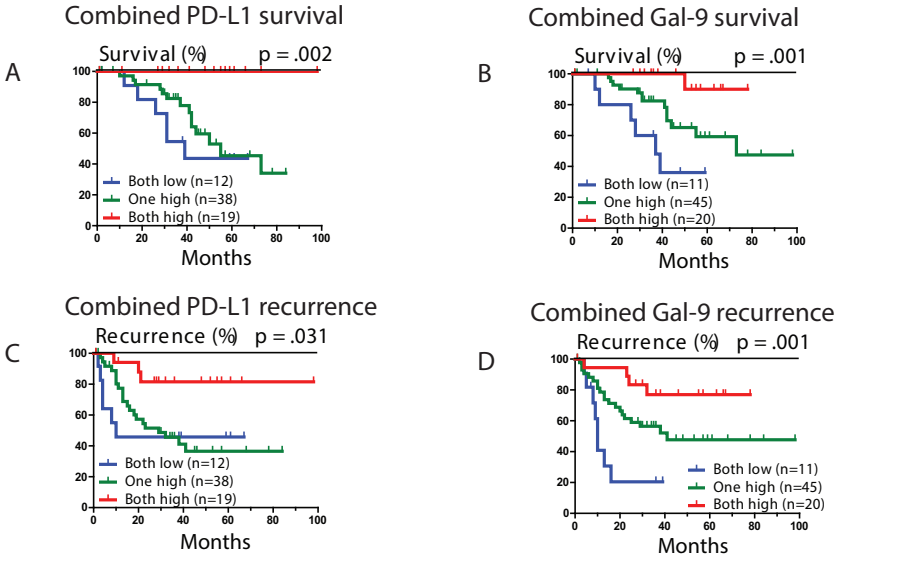

Figure 3. Kaplan-Meier graphs of combined circulating and intra-tumoral PD-L1 and Galectin-9. (A) HCC-specific mortality of combined circulating and intra-tumoral PD-L1. (B) HCC-specific mortality of combined circulating and intra-tumoral Gal-9. (C) Recurrence-free survival of combined circulating and intra-tumoral PD-L1. (D) Recurrence-free survival of combined circulating and intra-tumoral Gal-9.

Combining circulating and intra-tumoral expression

Given the lack of association between intra-tumoral expression and circulating levels of the respected ligands, and their independence for predicting survival in multivariate analysis, we examined if combining both improved the prognostication of patients with resected HCC. Figures 3A and 3B show the Kaplan-Meier survival curves of the combined (intra-tumoral and circulating) PD-L1 and Gal-9 biomarkers in relation to HCC death, respectively. Interestingly, patients with both high levels of circulating and intra-tumoral PD-L1 showed 100% survival (HR 0.33, 95%CI 0.16-0.68, p=.002). In addition, combined analysis of circulating and intra-tumoral Gal-9 could distinguish 3 groups of patients with distinct survival curves (HR 0.27, 95%CI 0.13-0.57, p=.001). Figures 3C and 3D show similar relationships of combined analysis of circulating and intra-tumoral PD-L1 and Gal-9 with HCC recurrence. In multivariate analysis both the PD-L1 combined biomarker and the Gal-9 combined biomarker, together with AFP > 100 μgl^{-1}, are independent predictors of HCC-specific survival (Table 2).

Table 2 Multivariate Cox proportional Hazard regression analysis of patients' survival

Variables	HR	95% CI	p-value
Size > 3cm	2.58	0.74-8.95	.136
Number of lesions	0.47	0.13-1.75	.260
Tumor differentiation	0.97	0.40-2.38	.953
Cirrhosis	3.25	1.10-9.60	**.033**
AFP > 100 μgl^{-1}	4.60	1.32-16.0	**.017**
Combined PD-L1	0.38	0.17-0.88	**.023**
Combined Gal-9	0.16	0.05-0.47	**.001**

DISCUSSION

We show that both circulating PD-L1 and Gal-9 are able to differentiate resected HCC patients prognostically. High levels of circulating PD-L1 and Gal-9 are associated with delayed recurrence and better survival of patients undergoing curative intent surgery for HCC. Interestingly, circulating levels of PD-L1 and Gal-9 were not correlated to intra-tumoral expression, and showed prognostic value independently of intra-tumoral expression. We also show that when intratumoral expression and circulating levels of PD-L1 and Gal-9 are combined prognostication improves even further.

No study to our knowledge has examined circulating PD-L1 in HCC. Circulating Gal-9 has not been studied in relation to survival in any cancer type. In contrast to our findings in HCC patients, high levels of circulating PD-L1 have been shown to be associated with

worse survival in renal cell cancer (35), diffuse B-cell lymphoma (36) and lung cancer (37). On the other hand, in agreement with our study, high levels of circulating PD-L1 have been shown to be associated with better survival in gastric adenocarcinoma (38). It is thus possible that the prognostic significance of these circulating biomarkers is tumor specific.

This study focused on the circulating levels of PD-L1 and Gal-9. In contrast, intra-tumoral or peri-tumoral protein expression of PD-L1 and Gal-9 in HCC have been examined in several studies before (16-23, 29, 34). Indeed our group has previously studied the prognostic value of intra-tumoral expression of PD-L1 and Gal-9 (24). While our current results regarding intra-tumoral expression of PD-L1 and Gal-9 (Figure 2) are in agreement with our previous observations, this agreement was expected given the significant overlap in patients between the two studies. Specifically, 58 patients of our previous cohort of 154 resected HCC patients had stored peripheral blood and were thus included in the current study. To these patients, an additional 23 patients were added. However, despite the significant smaller size of the current cohort we show, again, that high intra-tumoral expression of PD-L1 and Gal-9 are associated with improved survival.

The paradoxical observation that high intra-tumoral levels of immune inhibitory molecules are related to better, rather than worse, survival has been noted before and it is attributed to the phenomenon of adaptive immune resistance. Specifically, it has been observed that immune inhibitory molecules can be overexpressed on tumor cells and hepatocytes in response to IFN-γ or lymphocytic infiltration (44-47). Our observation that high circulating levels of PD-L1 and Gal-9 are also associated with better survival, may potentially be attributed to the same principle, namely that the presence of these molecules in the blood may signify active anti-tumor immunity.

Our original hypothesis was that circulating PD-L1 and Gal-9 would represent molecules that are passively released from tumor cells and therefore would reflect intra-tumoral expression of these molecules. However, similar to a recent study in B-cell lymphoma (36), we found that circulating levels of both PD-L1 and Gal-9 were not correlated to intra-tumoral expression. In addition, circulating PD-L1 and Gal-9 levels, in the current study, did not correlate with their expression on hepatocytes in tumor-free liver tissues. Instead, we found that circulating levels and intra-tumoral expression contributed independently to prognostication. It is thus possible that intra-tumoral and circulating forms of PD-L1 and Gal-9 have different pathophysiologic origins and/or functions.

One other hypothesis for the source of circulating PD-L1 is that it can be actively shed from membrane-bound PD-L1 expressing tumor cells and/or hepatocytes. Soluble PD-

L1 has indeed been shown to be released into cell culture supernatants by several, but not all, membrane PD-L1 expressing tumor cell lines (35), suggesting that expression of soluble and membrane-bound PD-L1 are differentially regulated. Another possibility, is that it is released from tumor associated macrophages. It is known that tumor associated macrophages and Kupffer cells express high levels of PD-L1 in the tumor microenvironment in HCC (17, 21, 23) and myeloid cells, especially mature dendritic cells, have been shown to release soluble PD-L1 *in vitro* (48). Moreover, it has been recently shown that circulating activated macrophages in ovarian cancer patients also express high levels of PD-L1 (49, 50). Whatever the source of circulating PD-L1 may be, the circulating ligand is known to retain its PD-1 binding domain and immunosuppressive properties (35).

Like PD-L1, Gal-9 is not only expressed on tumor cells, but also at high levels on tumor-associated macrophages in HCC (29). Gal-9 does not have a signal peptide, and its secretion must involve a non-classical pathway. Cleavage from the cell surface by matrix-metalloproteinases has been suggested as a secretion pathway (51), while other studies showed that cancer cells can secrete Gal-9 via exosomes (52). Therefore, circulating levels of these molecules are probably not passive reflections of tissue expression. In addition, while their expression in tumor tissues probably determines their local effects, their circulating counterparts may exert systemic effects, e.g. by inhibiting systemic immunity or, in case of Gal-9, prevent formation of distant metastasis by hampering extravasation of tumor cells into other tissues (32, 33). This means that future studies on the role of PD-L1 and Gal-9 as prognostic or predictive markers should take both intra-tumoral and circulating levels into consideration to maximize the potential for biomarker optimization.

Our study has several strengths. It is the first to study circulating levels of PD-L1 and Gal-9 in HCC. In addition, few studies have studied circulating levels of these molecules in other cancers, thus this is a fairly unexplored field of research. Most importantly both the intra-tumoral expression and the circulating levels of these molecules were studied. The observation that intra-tumoral and circulating levels do not correlate, but may independently contribute to prognostication, is novel. Testing for the expression of these molecules in the surrounding TFL tissue also allowed us to show that it is not the expression in the diseased liver that determines circulating levels of PD-L1 and Gal-9. Our study has also limitations. Stored peripheral blood samples were only available from 81 patients, therefore independent validation of these results is required. Given the importance of developing optimal immune specific biomarkers in the "era" of immunotherapy additional studies should confirm or refute these findings.

In summary, circulating levels of PD-L1 and Gal-9 do not correlate to their expression in tumor tissue, but have prognostic value in HCC patients independently of their expression in tumor tissue. Combined circulating levels and intra-tumoral expression of PD-L1 and Gal-9 are associated with more confident prognostic immune biomarker profiles.

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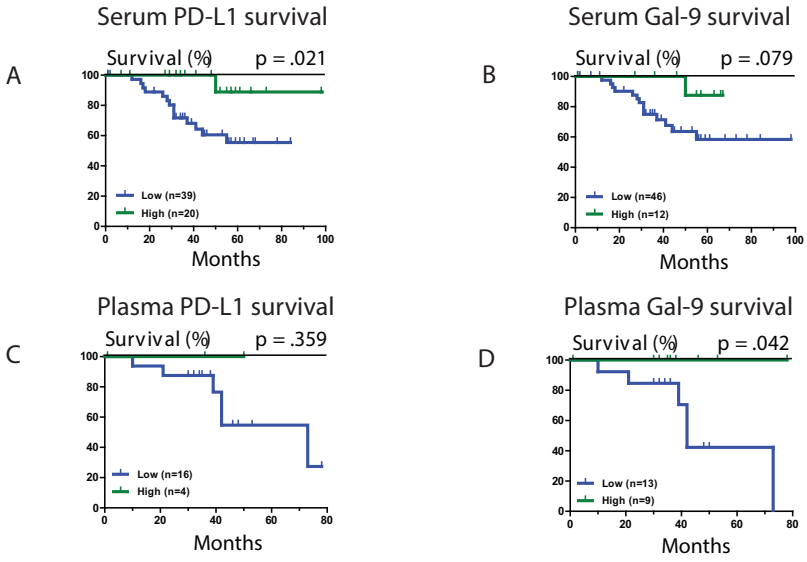
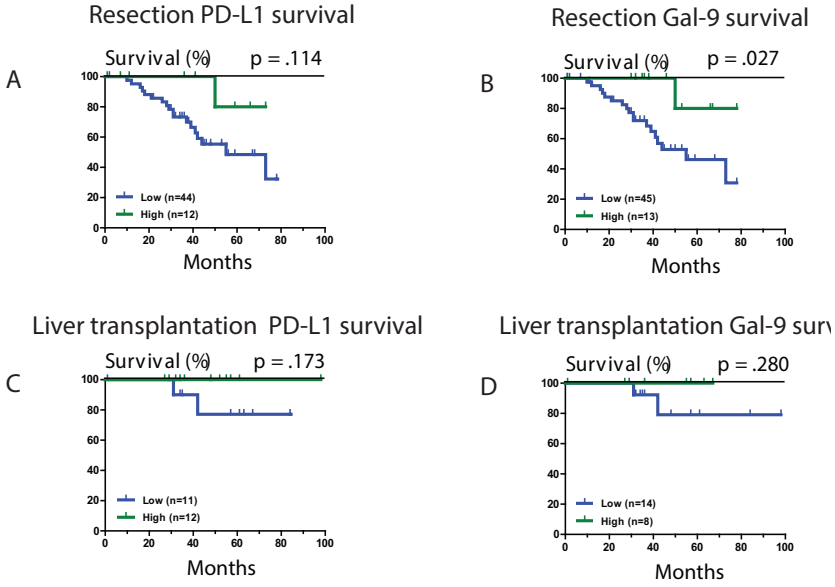
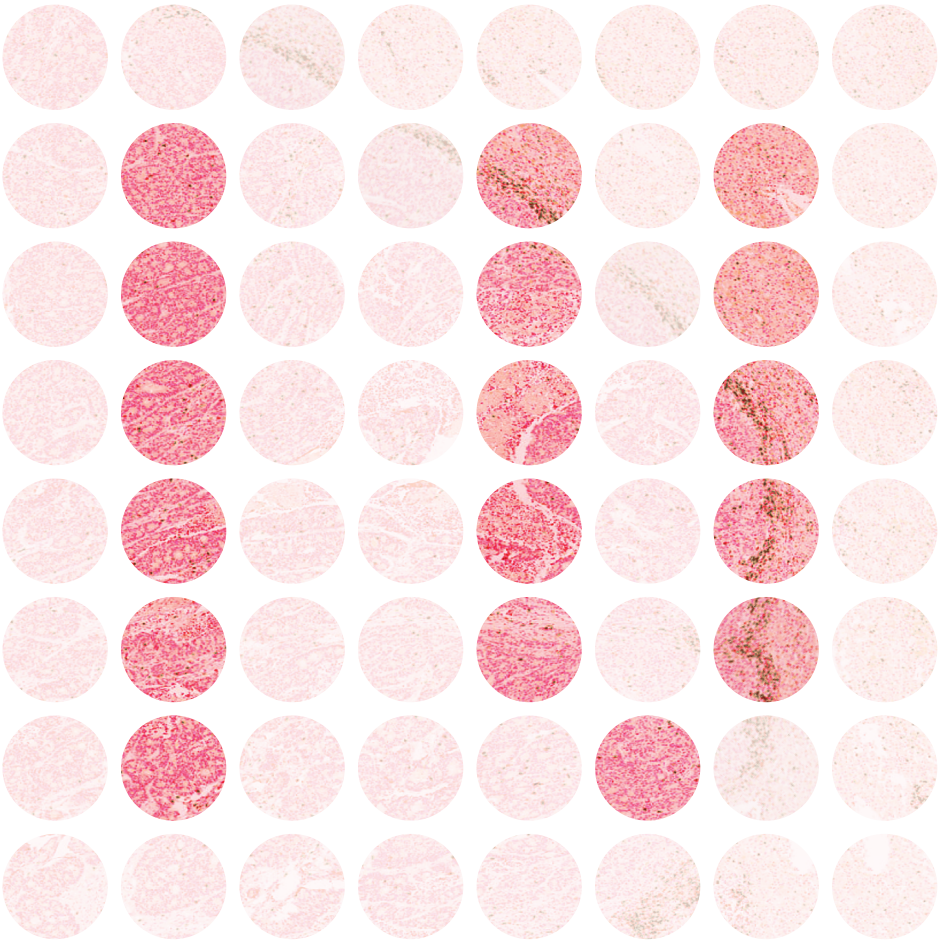
Supplementary Figures



Supplementary Figure 1: Kaplan-Meier graphs of circulating PD-L1 and Galectin-9 in patients with serum versus plasma. (A) HCC-specific mortality in relation to serum PD-L1 concentration. (B) HCC-specific mortality in relation to serum Gal-9 concentration. (C) HCC-specific mortality in relation to plasma PD-L1 concentration. (D) HCC-specific mortality in relation to plasma Gal-9 concentration.

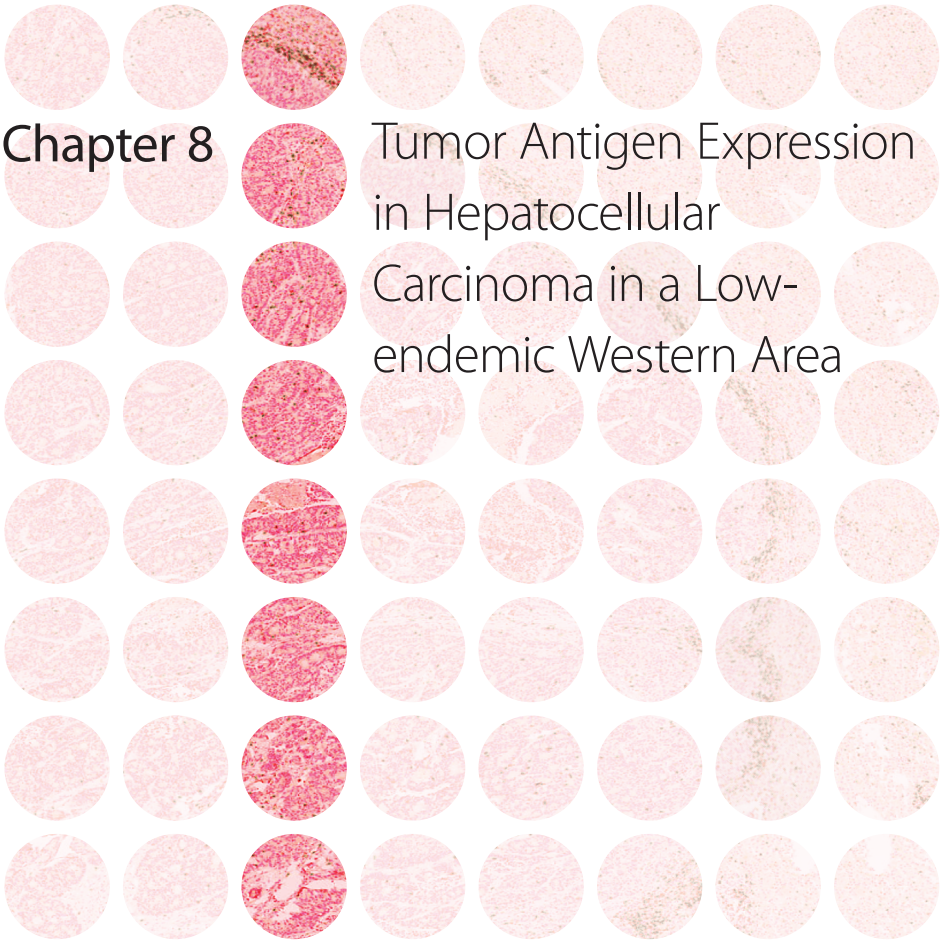


Supplementary Figure 2: Kaplan-Meier graphs of circulating PD-L1 and Galectin-9 in patients with resection versus liver transplantation. (A) HCC-specific mortality in relation to PD-L1 concentration of patients with resection. (B) HCC-specific mortality in relation to Gal-9 concentration of patients with resection. (C) HCC-specific mortality in relation to PD-L1 concentration of patients with liver transplantation. (D) HCC-specific mortality in relation to Gal-9 concentration of patients with liver transplantation.



PART IV

TUMOR ASSOCIATED ANTIGEN EXPRESSION



Chapter 8

Tumor Antigen Expression in Hepatocellular Carcinoma in a Low-endemic Western Area

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ABSTRACT

Introduction: Identification of tumor-antigens is crucial for the development of vaccination strategies against hepatocellular carcinoma (HCC). Most studies come from eastern-Asia, where hepatitis-B is the main cause of HCC. However, tumor-antigen expression is poorly studied in low-endemic, western, areas where the etiology of HCC differs.

Methods: We constructed tissue-microarrays from resected HCC tissue of 133 patients. Expression of a comprehensive panel of cancer-testis (MAGE-A1, MAGE-A3/4, MAGE-A10, MAGE-C1, MAGE-C2, NY-ESO-1, SSX-2, sperm protein 17), onco-fetal (AFP, Glypican-3) and over-expressed tumor-antigens (Annexin-A2, Wilms tumor-1, Survivin, Midkine, MUC-1) was determined by immunohistochemistry.

Results: A higher prevalence of MAGE antigens was observed in patients with hepatitis-B. Patients with expression of more tumor antigens in general had better HCC specific survival (p=.022). The 4 tumor-antigens with high expression in HCC and no, or weak, expression in surrounding tumor-free-liver tissue, were Annexin-A2, GPC-3, MAGE-C1 and MAGE-C2, expressed in 90%, 39%, 17% and 20% of HCCs, respectively. Ninety-five percent of HCCs expressed at least one of these 4 tumor-antigens. Interestingly, GPC-3 was associated with SALL-4 expression (p=.001), an oncofetal transcription-factor highly expressed in embryonal stem-cells. SALL-4 and GPC-3 expression were correlated with vascular-invasion, poor-differentiation and higher AFP levels before surgery. Moreover, patients who co-expressed higher levels of both GPC-3 and SALL-4 had worse HCC-specific survival (p=.018).

Conclusion: We describe a panel of 4 tumor-antigens with excellent coverage and good tumor specificity in a western area, low-endemic for hepatitis-B. The association between GPC-3 and SALL-4 is a novel finding and suggests that GPC-3 targeting may specifically attack the tumor stem-cell compartment.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related death with over half a million deaths per year worldwide (El-Serag et al., 2001; Jemal et al., 2011). HCC is more prevalent in Eastern Asia (Jemal et al., 2011) where hepatitis-B (HBV) accounts for 65% of HCC cases (Perz et al., 2006). In contrast, Western Europe is a low-endemic area where HBV is not the main cause of HCC, (Perz et al., 2006), and HCC is often diagnosed in non-cirrhotic livers (Verhoef et al., 2004; Witjes et al., 2012). However, it is estimated that the incidence of HCC is expected to continue to rise significantly in Western Europe and North America due to the hepatitis C virus infections during the 1960's and 1970's (IARC, 2011).

Primary treatment for early stage disease includes resection, local ablation and, in selected cases, liver transplantation. However, only 20% of patients are candidates for curative procedures (El-Serag et al., 2008). Once the cancer is advanced cure is no longer possible and median survival is a dismal 6-8 months, which can be extended to 10-13 months with the addition of sorafenib, a tyrosine kinase inhibitor (Abou-Alfa et al., 2010; Llovet et al., 2008).

Recognition of the important role of the immune system in cancer surveillance and elimination (Zou, 2005) has led to the development of various immunotherapeutic strategies against cancer (Mellman et al., 2011). One such strategy, cancer vaccination, holds great promise, as has been recently demonstrated in prostate cancer (Kantoff et al., 2010a; Kantoff et al., 2010b). In HCC, cancer vaccine trials have shown promising results, in particular after local therapy to prevent relapses (Kuang et al., 2004; Lee et al., 2005; Peng et al., 2005a).

However, despite the promise of cancer vaccines, success has been limited due to a number of factors. One of these is the proper identification of tumor antigens. Important requirements for inclusion of tumor antigens in therapeutic vaccines are immunogenicity, prevalence of expression within the cancer population, tumor tissue specificity, and biologic significance (Cheever et al., 2009; Kvistborg et al., 2013; Lang et al., 2009). Multiple studies have described expression of tumor antigen panels in HCC but the vast majority of these studies were conducted in east Asian populations (Chen et al., 2001; Liang et al., 2013; Luo et al., 2002; Nakamura et al., 2006; Peng et al., 2005b; Sera et al., 2008; Shirakawa et al., 2009; Xia et al., 2013; Yan et al., 2011; Yorita et al., 2011) where the etiology of HCC is predominately related to HBV. Very few such studies have been performed in western, low-endemic areas (Riener et al., 2009).

Tumor tissue specificity refers to the predominant, most preferably exclusive, expression of the tumor antigen in cancer and not in normal tissues (Kvistborg et al., 2013). A strict interpretation of this requirement would limit tumor antigens to antigens resulting from somatic mutations, chromosomal translocations resulting in neo-antigens, or viral derived antigens. However, exome sequencing has recently shown that somatic mutation patterns in HCC are strongly variable between individual patients and therefore not suitable for design of off-the-shelf therapeutic vaccines (Fujimoto et al., 2012). The most promising alternative tumor antigens are cancer-testis antigens, which are exclusively expressed in germ cells but not in other normal tissues (Hofmann et al., 2008), and oncofetal antigens, expressed primarily during embryogenesis but not broadly in adult humans (AFP, Glypican-3). Both types of antigens are aberrantly expressed in various types of cancer. In addition, self-antigens that are overexpressed in cancer (Survivin, Wilms tumor-1, Midkine, Annexin-A2) are also considered tumor antigens, and several clinical trials are underway in HCC and other cancers, targeting these types of overexpressed self-antigens. Many of the existing studies in HCC do not include tumor antigen expression in the corresponding surrounding tumor free liver (TFL) compartment and thus tissue specificity cannot be assessed.

In this study we used immunohistochemistry on tissue-microarrays (TMAs) to examine on the protein level the expression pattern in HCC of a comprehensive panel of 15 tumor antigens belonging to different categories, including the cancer testis antigens MAGE-A1, MAGE-A3/4, MAGE-A10, MAGE-C1, MAGE-C2, NY-ESO-1, Sperm Protein 17 (SP17) and SSX-2, the oncofetal proteins AFP and Glypican-3 (GPC-3), the over-expressed tumor antigens Annexin-A2, Wilms tumor-1 (WT-1), Survivin, Midkine (MDK) and the glycoprotein MUC-1. All these antigens have previously demonstrated immunogenicity in human studies. In addition, we tested for the expression of SALL-4, a transcription factor involved in the maintenance of embryonic and cancer stem cells (Zeng et al., 2014). SALL-4 has recently been shown to be expressed in an HCC subtype with stem-cell like features and to be associated with poor prognosis (Oikawa et al., 2013; Yong et al., 2013b; Zeng et al., 2014). The goal of the study was to identify a panel of biologically relevant tumor antigens with a) broad expression in a Western European population of HCC patients and b) specific expression in the tumor tissue with no, or little, expression in surrounding TFL tissue.

MATERIALS AND METHODS

Patient population and tissue samples

Archived formalin fixed paraffin-embedded tissue samples from 133 patients who underwent hepatic resection (n=94) or liver transplantation (n= 39) for HCC in our center,

between July 2004 and October 2013, were used for this study. Clinicopathologic characteristics are shown in Table 1. All patients had undergone procedures with curative intent and none had received systemic therapy prior to resection or transplantation. Patients with evidence of residual cancer after resection were excluded. Informed consent for the use of tissue for research purposes was obtained from all patients.

TMA construction

Three 0.6mm cores were taken from the tumorous area of 133 patients and two 0.6mm cores were taken from the corresponding TFL tissue of 105 of these patients. The tumorous as well as the TFL areas with vital tissue were marked by an experienced pathologist using archived H&E glass slides. In each TMA we included cores of testis, placenta, tonsil, ovary, stomach, prostate, bladder, kidney, lung and liver as control tissues. The TMAs were made using a Beecher© automated tissue-arrayer ATA-27 (Beecher Instruments, Sun Prairie WI, USA).

Immunohistochemistry

Immunohistochemistry was performed on 4μm thick sections mounted on Superfrost Plus™ slides (Erie Scientific LLC, Portsmouth NH, USA). The sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 15 minutes. Antigen retrieval was performed in a microwave for 10 minutes using the appropriate antigen retrieval buffer for each antigen (table 2). After serum block, primary antibodies were applied at 4^c overnight. The primary antibodies (Table 2) were carefully selected to be monoclonal (with the exception of AFP and SP17) and to have been validated in scientific literature. HRP-conjugated anti-mouse or anti-rabbit polymer secondary antibody (Envision™, DAKO, Glostrup, Denmark) was then applied for 1 hour, followed by diaminobenzadine (DAB) as the chromogen detection method. The slides were stained with haematoxylin followed by dehydration. The above protocol was used for all antibodies with the exception of GPC-3 and AFP where an automated BenchMark ULTRA™ instrument (Ventana Medical Systems, Inc, Tuscon AZ, USA) was used in a clinical laboratory setting. Scoring was performed by 2 independent investigators and differences resolved by mutual agreement. Intensity was scored as either none, weak, moderate or strong, while percentage of positive cells was scored as <5%, 5-25%, 25-75% and >75%. For a staining to be considered positive at least 5% of cells had to be stained. Negative controls consisted of omission of the primary antibody and appropriate positive control tissues were used for all antibodies. H-scores were calculated by multiplying the intensity score (0 to 3) with the level of % of positive cells where 1 = <5%, 2 = 5-25%, 3 = 25-75% and 4 = >75%.

Statistical analysis

The association of the expression level of tumor antigens with the various subgroup populations was analyzed using the Chi-square test. The association of the tumor

Table 1. Patient characteristics

Characteristics	No. of patients 133 (%)
Age (years)	
Median	60.4
Range	22.9-86.6
Gender	
Male	95 (71.4)
Female	38 (38.6)
Ethnicity	
Western-European	103 (77.4)
Non western-European[a]	30 (22.7)
Etiology[b]	
No known liver disease	37 (27.8)
Hepatitis B	24 (18.0)
Alcohol abuse	22 (16.5)
Hepatitis C	18 (13.5)
Cryptogenic	10 (7.5)
NASH	9 (6.8)
Hemochromatosis	5 (3.8)
Primary biliary cirrhosis	3 (2.3)
Other	5 (3.8)
Viral hepatitis status[c]	
Hepatitis B positive[d]	30 (22.6)
Hepatitis C positive[e]	19 (14.3)
Cirrhosis present	
Yes	69 (51.9)
No	64 (48.1)
Tumor differentiation	
Good	44 (31.4)
Moderate	73 (52.1)
Poor	23 (16.4)
Vascular invasion	
Yes	71 (62.3)
No	47 (37.7)
Number of lesions	
Single	90 (67.7)
Multiple	43 (32.3)
Size of largest lesion	
Median	4.5cm
Range	0.5-25
AFP level before resection	
Median	8ug/l
Range	1-63,000

← **Table 1.** Patient characteristics

[a] Non-western European patients are from East-Europe (n=3), Suriname (n=7), Middle-East (n=8), Sub-Sahara Africa (n=3) and South-East Asia (n=9). See Supplementary Table 1.

[b] Patients with more than 1 etiologic factor were listed based on the most dominant cause of liver disease.

[c] Three patients had both hepatitis B and hepatitis C.

[d] HBsAg(+) and/or anti-HBc positive, [e] anti-HCV positive

antigen expression with the clinicopathologic parameters was analyzed using the Chi-square tests for categorical variables and the student T-test for continues variables. Survival analyses was performed using the Kaplan–Meier method and the log-rank test. Univariate and multivariate hazard ratios, 95% confidence intervals, and corresponding p-values were obtained using Cox regression analysis. The statistical analysis was performed using the SPSS© 21 software.

Table 2. Primary antibodies

Antigens	Primary antibody source	Clone	Retrieval buffer	Antibody dilution	References
MAGE-A1	Santa Cruz Biotechnology	MA454	Tris EDTA	1:50	(Jungbluth et al., 2000)
MAGE-A3/4	Prof. G.C. Spagnoli[a]	57B	Tris EDTA	1:100	(Landry et al., 2000)
MAGE-A10	Prof. G.C. Spagnoli[a]	3GA11	Citric acid	1:10	(Schultz-Thater et al., 2011)
NYESO-1	Santa Cruz Biotech.	E978	Tris EDTA	1:50	(Vaughan et al., 2004)
SSX-2	Prof. A.G. van Kessel[b]	E3AS	Tris EDTA	1:25	(dos Santos et al., 2000)
MAGE-C1	Santa Cruz Biotech.	CT7-33	Tris EDTA	1:50	(Xia et al., 2013)
MAGE-C2	Prof. Boquan Yin[c]	CT-10	Tris EDTA	1:100	(Zhuang et al., 2006)
MUC-1	Sanbio	MA695	Citric acid	1:100	(Langner et al., 2004)
AFP	Dako	Polyclonal	Tris EDTA	1:400	Dako[d]
GPC-3	Santa Cruz Biotech.	1G12	Tris EDTA	1:200	(Shirakawa et al., 2009)
Annexin-A2	BD Biosciences	5	Tris EDTA	1:200	(Yee et al., 2007)
WT-1	Novus Biologicals	6F-H2	Tris EDTA	1:400	(Nakatsuka et al., 2006)
Survivin	Santa Cruz Biotechnology	D-8	Tris EDTA	1:50	(Brennan et al., 2008)
MDK	GeneTex	EP1143Y	Citric acid	1:400	(Liang et al., 2013)
SP17	Proteintech	Polyclonal	Citric acid	1:100	Proteintech[e]
SALL-4	Santa Cruz Biotech.	EE-30	Tris EDTA	1:50	(Yong et al., 2013b)

[a] MAGE-A3/A4 and MAGE-A10 antibodies graciously provided by Professor Giulio Spagnoli, Department of Surgery, Research Laboratory, University Hospital Basel, Basel, Switzerland (Landry et al., 2000; Schultz-Thater et al., 2011)

[b] SSX-2 antibody graciously provided by Professor Ad Geurts van Kessel, Department of Human Genetics University Hospital Nijmegen, 6500 HB Nijmegen, The Netherlands (dos Santos et al., 2000)

[c] MAGE-C2 antibody graciously provided by Professor Boquan Yin, Department of Immunology, Fourth Military Medical University, Xi'an 710032, PR China (Zhuang et al., 2006)

[d] http://www.dako.com/nl/ar38/p102130/prod_products.htm Accessed 8-9-14

[e] http://www.ptglab.com/PView/SPA17-Antibody-13367-1-AP-PVIEW.htm Accessed 8-9-14

RESULTS

Tumor antigen expression in HCC and TFL tissue

The expression of the 15 tumor antigens in both tumor and TFL tissue is shown in Table 3. No expression of SSX-2 and MUC-1 was observed, although the antibodies properly stained testis (seminiferous duct cells) and gastric control tissue respectively. The prevalence of expression of MAGE-A3/4, NY-ESO-1, AFP, MAGE-A1 and MAGE-A10 was low (< 10% of patients), while increasing numbers of HCC showed expression of MAGE-C1, MAGE-C2, GPC-3, MDK, Survivin, WT-1, SP17 and Annexin-A2 (prevalence ranging from 17% to 90% - Table 3). However, the overexpressed self-antigens MDK, Survivin, WT-1 and SP17 showed equal expression in tumors and in TFL tissues. Thus, the tumor antigens with the highest differential expression level between tumor tissue and TFL tissue are Annexin-A2 (90.2% vs 37.1), GPC-3 (39.1% vs 0%), MAGE-C2 (19.5% vs 0%) and MAGE-C1 (17.3% vs 0%). This conclusion did not change when we analyzed only the 105 patients with paired tumor and TFL tissue as compared to the entire cohort of 133 patients. Representative immunohistochemical stainings of these four tumor antigens in HCC and TFL tissue are shown in Figure 1, while representative immunohistochemical stainings of all the tumor antigens can be seen in Supplementary Figure 1. The distribution of intensity and the percentage of stained cells in tumor tissue, and in the case of Annexin-A2 in TFL tissue, is shown in Figure 2. MAGE-C1 and GPC-3 showed cytoplasmic expression in tumor cells, while MAGE-C2 showed nuclear expression in tumor cells.

Table 3. Tumor antigen expression

Antigens	% positive stainings in tumor tissue (n=133)	% positive stainings in TFL tissue (n=105)
SSX-2	0	0
MUC-1	0	0
MAGE-A3/4	3.0	0
NYESO-1	3.8	0
AFP	6.8	0.9
MAGE-A10	7.5	0
MAGE-A1	9.8	0
MAGE-C1	17.3	0
MAGE-C2	19.5	0
GPC-3	39.1	0
MDK	57.7	64.4
Survivin	79.5	91.1
WT-1	85.6	84.6
SP17	87.0	88.0
Annexin-A2	90.2	37.1

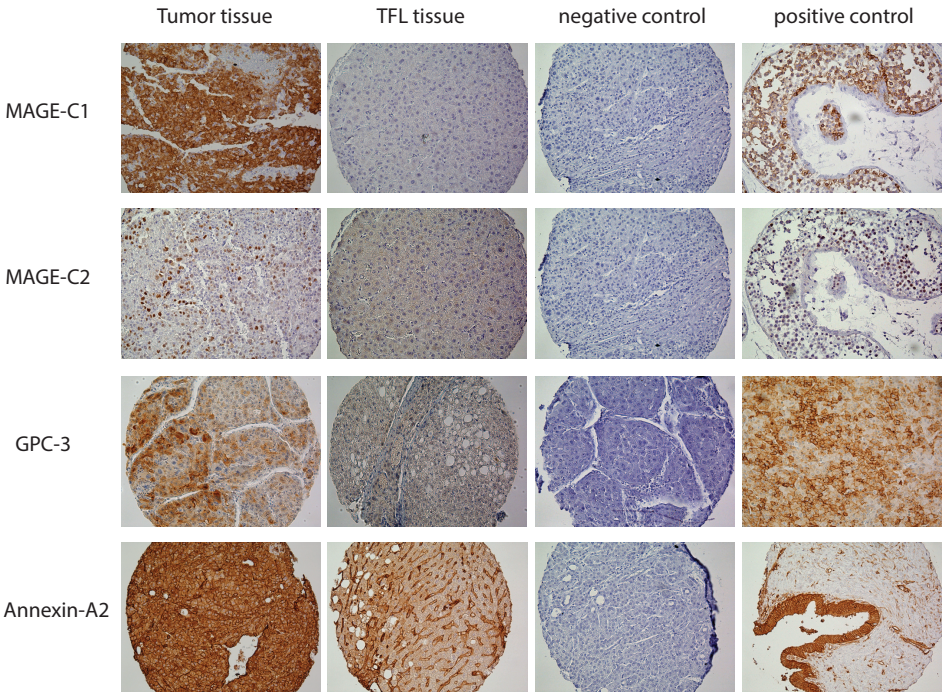


Figure 1. Representative stainings for tumor tissue and TFL tissue with negative and positive controls for MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2. Strong tumor cell stainings for MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2 are seen in the leftmost column. The second column shows lack of staining in the corresponding TFL tissues with the exception of Annexin-A2 where staining of sinusoids is seen. The third column shows the corresponding negative controls and the last column shows the corresponding positive controls which are testis tissue for MAGE-C1 and MAGE-C2, fetal liver tissue for GPC-3 and pancreatic cancer tissue for Annexin-A2.

Annexin-A2 showed membranous and cytoplasmic expression in hepatocytes in HCC and TFL tissue, and stained sinusoidal endothelium. These expression patterns are in agreement with previous observations in HCC (Liang et al., 2013; Longerich et al., 2011; Riener et al., 2009). Only hepatocyte and not sinusoidal staining was scored for Annexin-A2. Moreover, Annexin-A2 expression showed a weaker intensity in the hepatocytes of the TFL tissue than the corresponding tumor cells (Figure 2D and E). Looking at etiologic factors, there was a significantly higher prevalence of expression of MAGE-A3/4 (p=.011), MAGE-A1 (p=.034) and MAGE-C1 (p=.008) in patients with HBV infection compared to patients without HBV infection, while MAGE-C2 (p=.264) and GPC-3 (p=.334) showed a statistical trend towards higher expression in patients with HBV infection (Figure 3).

Tumor antigen index.

As in previous studies (Liang et al., 2013) a tumor antigen index (TAA) was calculated based on the total number of antigens co-expressed in a given tumor tissue. Patients

were grouped based on whether they co-expressed 0-2 tumor antigens, 3-6 tumor antigens or 7-9 tumor antigens. No patients co-expressed more than 9 out of the 15 tumor antigens. The higher the TAA index the better the HCC-specific survival was (p=.020, Supplementary Figure 2). In multivariable analysis this was an independent prognostic factor for HCC-specific survival (Table 4).

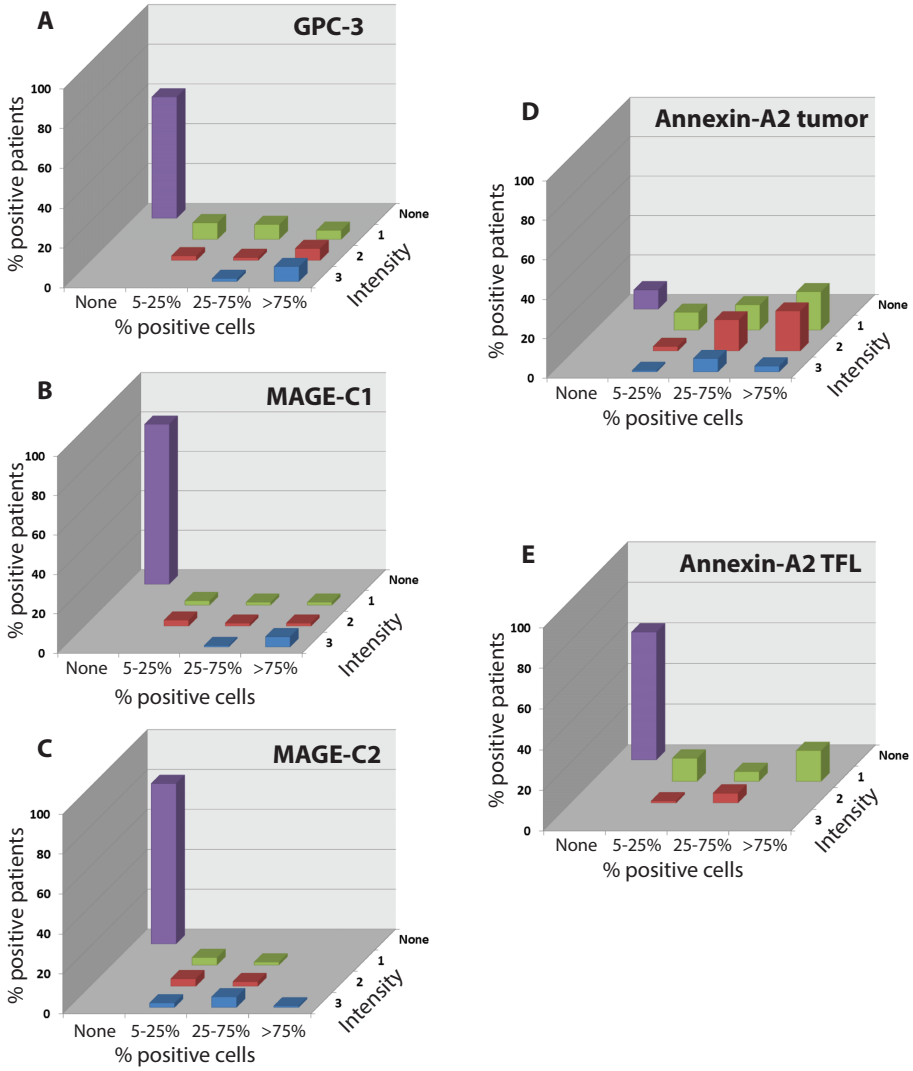


Figure 2. Distribution of staining intensity and percentage of positive cells for MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2. GPC-3 cancer staining (A), MAGE-C1 cancer staining (B), MAGE-C2 cancer staining (C), Annexin-A2 cancer staining (D), Annexin-A2 TFL staining (E). Intensity 1=weak; 2=moderate; 3=strong.

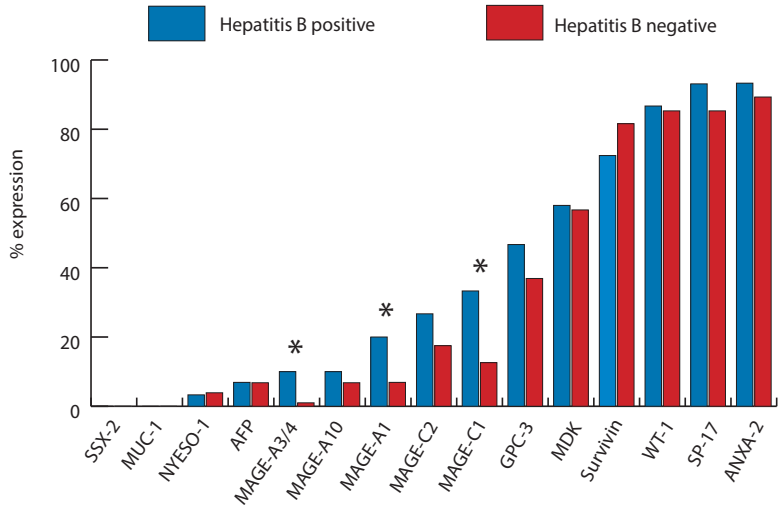


Figure 3. Antigen expression based on hepatitis B status. * = p<0.05.

Table 4. Cox proportional Hazard regression analysis of patients' overall survival

Variables		Univariate			Multivariate		
		HR	95% CI	p-value	HR	95% CI	p-value
AFP >400 ug/l		2.867	1.176-6.992	**.021**	2.682	0.947-7.601	.063
>3 vs ≤3 lesions		4.438	1.594-12.353	**.004**	3.771	1.276-11.141	**.016**
TAA index	0-2 vs 3-6 antigens	0.266	0.076-0.925	**.042**	0.238	0.062-0.909	**.033**
	0-2 vs 7-9 antigens	0.070	0.007-0.711		0.048	0.004-0.557	
High H-score for both GPC-3 and SALL-4		3.119	1.154-8.430	**.025**	3.674	1.120-12.055	**.032**

Co-expression patterns of MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2

Further analysis was performed on the 4 antigens with the greatest differences in expression between tumor and TFL tissue, namely MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2. Expression of at least 1 of these antigens was observed in tumor tissues of 95% of patients, while 48% of patients expressed only 1 antigen, 30% expressed 2 antigens, 11% expressed 3 antigens and 6% expressed 4 antigens (Figure 4a). Co-expression of these antigens in individual patients is shown in Figure 4b. Ninety-two percent of patients express, individually, or in combination, Annexin-A2 and GPC-3. Of the patients that do not express Annexin-A2 or GPC-3, 3% express both MAGE-C1 or MAGE-C2. In 11% of patients MAGE-C1 and MAGE-C2 add a second tumor antigen to patients that otherwise express only 1 antigen, either GPC-3 or Annexin-A2. Finally, in 10% of patients MAGE-C1 and MAGE-C2 add a third antigen to patients that co-express GPC-3 and Annexin-A2. Interestingly, MAGE-C1 and MAGE-C2 are significantly and strongly co-expressed in tumors of our HCC patients (p<.001, Pearson's correlation coefficient =

.68). This panel of 4 antigens also covers tumors of most HBV-negative patients (95%), of which 52% expressed 1 antigen, 29% expressed 2 antigens, 9% 3 antigens and 5% 4 antigens. Together, these data show that this panel of 4 tumor antigens may be suitable for vaccination studies in HCC patients in western low-endemic areas.

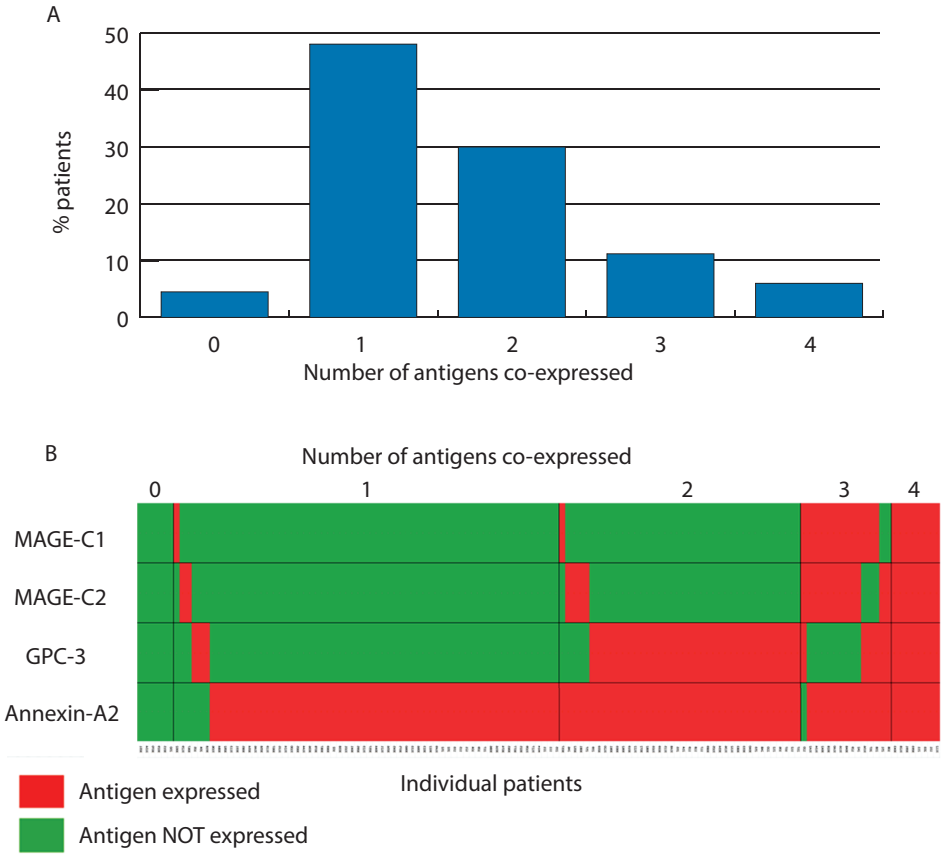


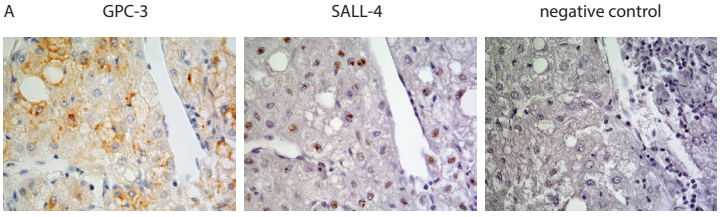
Figure 4. Co-expression of MAGE-C1, MAGE-C2, GPC-3 and Annexin-A2 antigens. Distribution of total number of antigens expressed in the tumors of HCC-patients (A). Heat-map representation of 133 individual patients with expression of each antigen per patient (B).

Expression of SALL-4 and co-expression with GPC-3

SALL-4 is a transcription factor involved in the maintenance of embryonic and cancer stem cells (Zeng et al., 2014) and has recently been shown to be expressed in an HCC subtype with stem-cell like features associated with poor prognosis (Oikawa et al., 2013; Yong et al., 2013b; Zeng et al., 2014). In our study SALL-4 nuclear expression was seen in 26% of tumors and in none (0%) of the TFL samples. Like in previous studies (Zeng et al., 2014), SALL-4 was more frequently expressed in tumors of patients with HBV infec-

tion (40% vs 22%, p=.05) and its expression was correlated with poor differentiation (p=.002) and higher AFP levels before surgery (p=.007), while there was a trend towards correlation with vascular invasion (p=.081). Interestingly, there was a significant correlation between SALL-4 expression and GPC-3 expression (p=.001, Pearson's correlation coefficient =.29, Figure 5). While neither SALL-4 or GPC-3 were individually associated with HCC-specific survival, there was a trend towards worse HCC-specific survival in patients co-expressing both GPC-3 and SALL-4 (p= .190, Supplementary Figure 3a). In addition, when the strength of the staining was taken into consideration in the form of the H-score (intensity x % of positive cells) patients who co-expressed high levels of both SALL-4 (H-score>2) and GPC-3 (H-score>3) had a significantly worse HCC-specific survival (p= .018, Supplementary Figure 3b). This was an independent prognostic factor in multivariate analysis (Table 4).

A



B

p=.001 Pearson's coefficient = .29		SALL-4		
		–	+	Total
GPC-3	–	68 (51.1%)	13 (9.8%)	81
	+	30 (22.5%)	22 (16.5%)	52
	Total	98	35	133

Figure 5. Co-expression of GPC-3 and SALL-4. Representative case co-expressing GPC-3 and SALL-4 (A). 2x2 table of expression status of GPC-3 and SALL-4 in the entire cohort (B).

Relationship of individual tumor antigen expression to known prognostic markers

Of all the tumor antigens tested, GPC-3 was the one most strongly associated with known prognostic factors. Specifically, GPC-3 was associated with poor tumor differentiation (p=.004), the presence of vascular invasion (p=.002), and higher AFP before resection (p=.03).

DISCUSSION

The aim of this study was to identify a panel of tumor antigens suited for immunotherapeutic approaches, such as vaccination, for HCC in western-European, low-endemic areas, where HBV infection is not the main etiology of HCC and where the diagnosis is often made in non-cirrhotic livers (Verhoef et al., 2004; Witjes et al., 2012). In our cohort, only 23% of patients were HBV positive, 14% HCV-positive and 48% had no liver cirrhosis. In addition 77.4% of our patients are of western-European decent. Supplementary Table 1 describes the hepatitis-B status of patients per patient region of origin. Groups other that western-European are too small for subgroup analysis of antigen expression.

The observed prevalence of expression of testis and oncofetal antigens was generally lower than previous studies reported. Most of these prior studies have been conducted in East Asia where HBV infection is the most prevalent cause of HCC and the majority of HCC patients have liver cirrhosis. For example, a previous East Asian study reported that 36% of patients expressed MAGE-C1 (Xia et al., 2013), while we found only 17% expression. Supporting the association between cancer testis antigens and HBV infection, we found increased prevalence of MAGE-A1, MAGE-A3/4, and MAGE-C1 expression in HBV-positive patients. The prevalence of MAGE-C1 expression in HCC tissues in our HBV positive patients was similar to that reported by Xia et. al. (32% in HBV positive versus 13% in HBV negative patients). The only other large western study that has examined several of these antigens by immunohistochemistry is by Riener et. al.(Riener et al., 2009) who studied 146 HCC patients from Switzerland, of which only 12% had HBV. In that study MAGE-C1 expression was found expressed in 12% of patients, NY-ESO-1 in 2% and MAGE-A3/4 in 0%, results which are similar to our findings. Likewise, expression of the oncofetal protein GPC-3 was found in 61-84% of patients in four Asian studies with HBV positivity ranging between 25-85% (Liang et al., 2013; Shirakawa et al., 2009; Yan et al., 2011; Yorita et al., 2011), and all these studies showed evidence of increased GPC-3 expression in the HBV positive patients compared to the HBV negative patients. In our study GPC-3 expression was found in 39% of all patients but in 48% of HBV-positive patients.

Another explanation for the relatively low prevalence of tumor antigen expression observed in our study is that many prior studies have used RT-PCR measuring mRNA expression (Chen et al., 2001; Luo et al., 2002; Peng et al., 2005b), while we have measured protein expression by immunohistochemistry. In fact, large discrepancies between tumor antigen expression in HCC by RT-PCR and immunohistochemistry have been reported. For example, Nakamura et. al.(Nakamura et al., 2006) found 18/41 of HCC samples (43%) expressing NY-ESO-1 by RT-PCR while only 3 (7%) expressed the protein.

It is likely that protein expression rather than RNA expression is a reliable predictor of suitability of tumor antigens for vaccination studies.

While the absence of MUC-1 expression in HCC is in agreement with previous work (Cao et al., 1999), the absence of SSX-2 in our study (0%) is in contrast to the study by Liang J et. al.(Liang et al., 2013) where a prevalence of 75% was reported. The use of different antibody clones may be one explanation. Clone 4D10, used in the Liang J at. al. study, was not tested in TFL samples to examine tumor specificity. In addition, two studies (Luo et al., 2002; Wu et al., 2006) using RT-PCR have shown expression in 2/21 and 13/36 HCC-patients respectively, indicating that it is unlikely that the true protein expression level of SSX-2 in HCC is very high. Finally, despite the lack of staining in tumor or TFL tissue, antibody clone E3AS, which we used, showed proper staining of positive control seminiferous duct cells in testis tissue (Supp. Figure 1).

AFP was found to be expressed in few HCC samples (7%) in our study. While an incidence as low as 2% has been reported (Ferrandez-Izquierdo & Llombart-Bosch, 1987) most studies show expression of AFP in around 17-50% of HCC tumors (Brumm et al., 1989; Ganjei et al., 1988; Lau et al., 2002; Minervini et al., 1997; Tsuji et al., 1999). To ensure accuracy of AFP staining in our study AFP expression was determined twice, under clinical laboratory conditions (automated BenchMark ULTRA™ instrument), which yielded identical results. On further examination AFP expression was strongly correlated with serum AFP level before resection ($p<.001$). Of the patients with a serum AFP >400 ug/l 29% expressed AFP in their tumors versus only 3% in patients with a serum AFP <400ug/l ($p=.001$). This correlation of AFP serum levels with tumor AFP expression has been demonstrated before (Li et al., 2011). In our cohort, however, only 17% of our patients had an AFP value above 400ug/l. Thus, one possible explanation for the low incidence of AFP staining is the relative low number of patients with high serum AFP levels. Indeed, most contemporary series report high AFP serum levels (> 400ug/l) in 27 to 45% of patients undergoing resection (Liu et al., 2014; Ma et al., 2013; Wang et al., 2009).

While MDK, SP17, WT-1 and Survivin were expressed in the majority of tumors, we observed similar expression in adjacent TFL tissues, suggesting they might be unsuitable for vaccination studies in HCC due to lack of tumor-tissue specificity. Indeed these 4 antigens have been shown to be expressed in tissues other than cancer (Deguchi et al., 2002; Kannangai et al., 2005; Monma et al., 2013; Scharnhorst et al., 2001), or other than cancer and testis in the case of SP17 (Frayne & Hall, 2002; Lacy & Sanderson, 2001). Although in some reports the expression of MDK, SP17 and WT-1 has been shown to be lower in TFL tissue than in HCC tissue (Koide et al., 1999; Sera et al., 2008; Xia et al., 2013;

Zhu et al., 2013), in the case of Survivin another report corroborates the equal or higher TFL, compared to tumor, expression (Chau et al., 2007).

It has been previously shown by Liang et.al. (Liang et al., 2013), that the higher the number of tumor antigens expressed by a given tumor the better the survival is. The hypothesis is that the higher the number of tumor antigens present the more the immunologic targets available to the immune system. In our study, in agreement with Liang et.al., we show that the higher the number of tumor antigens present in a given tumor the better the HCC-specific mortality is (table 4, Supplementary Figure 2). While our findings are supportive of the above hypothesis, further validation and experimentation is necessary to prove the concept.

Therapeutic vaccination with a panel of tumor antigens, as opposed to a single antigen, would have the advantage of better coverage of the target tumor cell population as well as covering patients who express different antigens in their tumors. The panel that we selected (MAGE-C1, MAGE-C2, GPC-3, Annexin-A2) covers 95% of patients, with nearly 50% of them expressing at least 2 antigens. In addition, since in many patients expression of each individual antigen is limited to only 5-25% of tumor cells (Figure 2), targeting multiple antigens per patient may be needed to realize a successful clinical outcome. In addition, our antigen panel lacks, for the most part, expression in TFL tissue, which is an advantage, since it may reduce unwanted side effects. Even in the case of Annexin-A2, where a sizable proportion of TFL samples expressed the antigen (37%), the level of expression was much lower than that in the corresponding tumor samples (Figure 2D and E).

Biologically, GPC-3, a heparin sulfate proteoglycan expressed during embryogenesis, has been shown to be a poor prognostic factor in multiple studies (Liang et al., 2013; Shirakawa et al., 2009; Yan et al., 2011; Yorita et al., 2011). We confirm that GPC-3 expression is associated with higher serum AFP level (Liang et al., 2013; Yorita et al., 2011), worse tumor differentiation (Shirakawa et al., 2009; Yan et al., 2011; Yorita et al., 2011), and the presence of vascular invasion (Yorita et al., 2011). The immunogenicity of GPC-3 has been well demonstrated, and a phase I clinical cancer vaccine trial has already demonstrated tolerability and biologic efficacy (Sawada et al., 2012). In addition, gene expression profiling has shown that GPC-3 is significantly overexpressed in CD90+ HCC stem cells (Ho et al., 2012), suggesting that targeting GPC-3 may enable eradication of the tumor stem cell compartment. Our newly reported association of GPC-3 with SALL-4 strengthens the notion that GPC-3 is involved in stem cell biology in HCC. In fact, we show that patients who co-express high levels of both GPC-3 and SALL-4 have worse HCC-specific survival (Supplementary Figure 3b), indicating that the co-expression is biologically significant.

However, it should be noted that strong co-expression is a relatively infrequent event occurring in 7.5% of patients. While other studies (Yan et al., 2011; Yong et al., 2013a) have shown worse overall survival for patients expressing individually GPC-3 or SALL-4 we did not show such an association. This is likely due to the fact that our study is smaller in size and was not designed to test the presence of biomarkers in HCC. In fact, when considering patients with higher GPC-3 staining, or patients with higher SALL-4 staining, statistical trends towards worse HCC-specific survival are apparent and consistent with the smaller size of our cohort (Supplementary Figures 3c, 3d). Finally, although both GPC-3 and SALL-4 are considered possible therapeutic targets in HCC (Filmus & Capurro, 2013; Yakaboski et al., 2014), information on immunogenicity of SALL-4 is lacking. Therefore, further research on immunogenicity of SALL-4 is needed before we can suggest to include SALL-4 in a therapeutic vaccine.

Annexin-A2, a calcium dependent phospholipid binding protein, is involved in membrane formation, exocytosis and interaction with the extracellular matrix (Gerke & Moss, 2002). It is overexpressed in HCC (Mohammad et al., 2008; Yu et al., 2007) and multiple other cancers (Zhang et al., 2012), is involved in invasion and metastasis (Zhao et al., 2010), and immunogenicity has been demonstrated (Liu et al., 2011; Zheng & Jaffee, 2012). Our results are in agreement with Liu et. al.(Liu et al., 2013) in that Annexin-A2 is expressed in the majority of patients with HCC and expression is significantly more pronounced in the tumor cells as compared to the surrounding TFL tissue. Our study is one of the very few to examine the protein level expression of Annexin-A2 in a "western" cohort. Longerich et. al.(Longerich et al., 2011) demonstrated Annexin-A2 expression in HCC in a small western European patient cohort, but did not study expression in TFL tissue.

MAGE-C1 and MAGE-C2 are involved in embryogenesis, their expression is known to be reactivated in various cancers, and they are known immunogens (Li et al., 2004). The strong co-expression between MAGE-C1 and MAGE-C2 was not surprising since the 2 genes are located close to each other on chromosome X (q27) and are likely translated together. However, despite their strong co-expression, a little less than half of positive cases expressed either one of the 2 antigens alone indicating a potential value in including both of these antigens in a tumor vaccine.

In conclusion, we show that there are etiological differences in tumor antigen expression in HCC. In addition, we describe a panel of 4 antigens, MAGE-C1, MAGE-C2, GPC-3 and Anexxin-A2, which combine several favorable characteristics for future vaccination studies in patients in western low-endemic areas, such as combined coverage for the majority of patients, as well as tumor specificity. Finally, we demonstrate for the first

time a relationship between GPC-3 and SALL-4 expression, which further substantiates that targeting GPC-3 may enable eradication of the HCC tumor stem-cell compartment.

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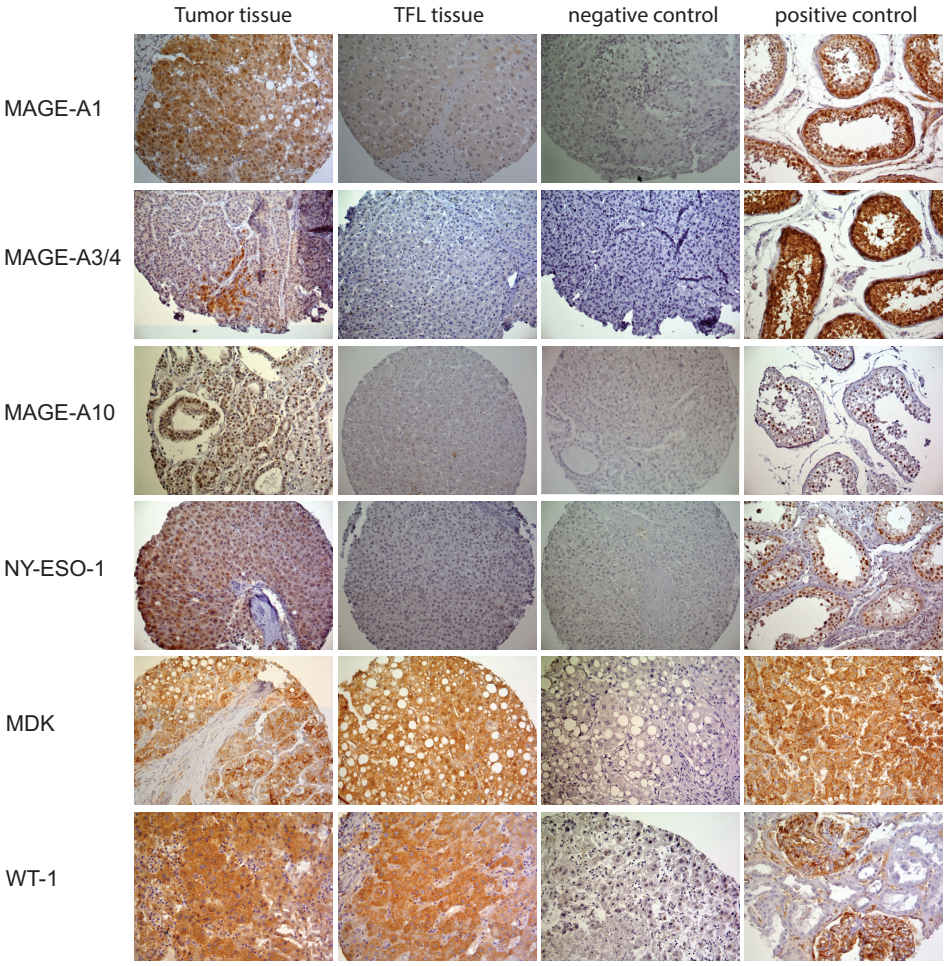
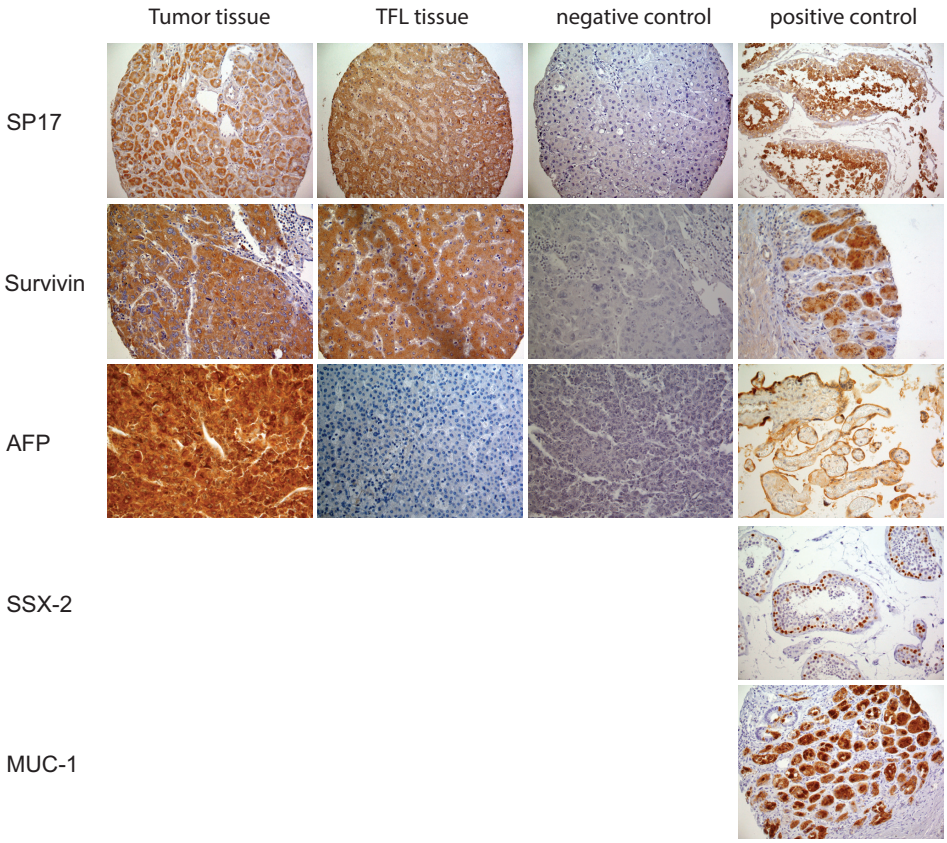
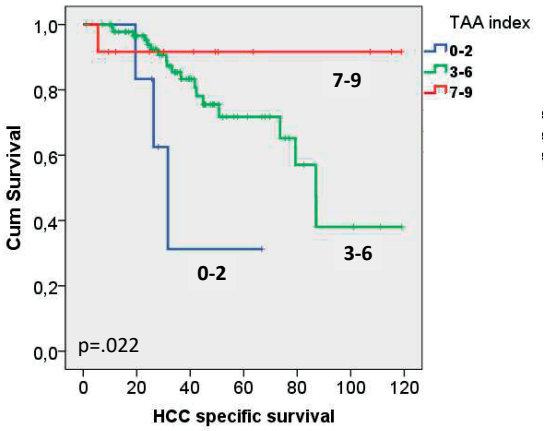
Supplementary Figures



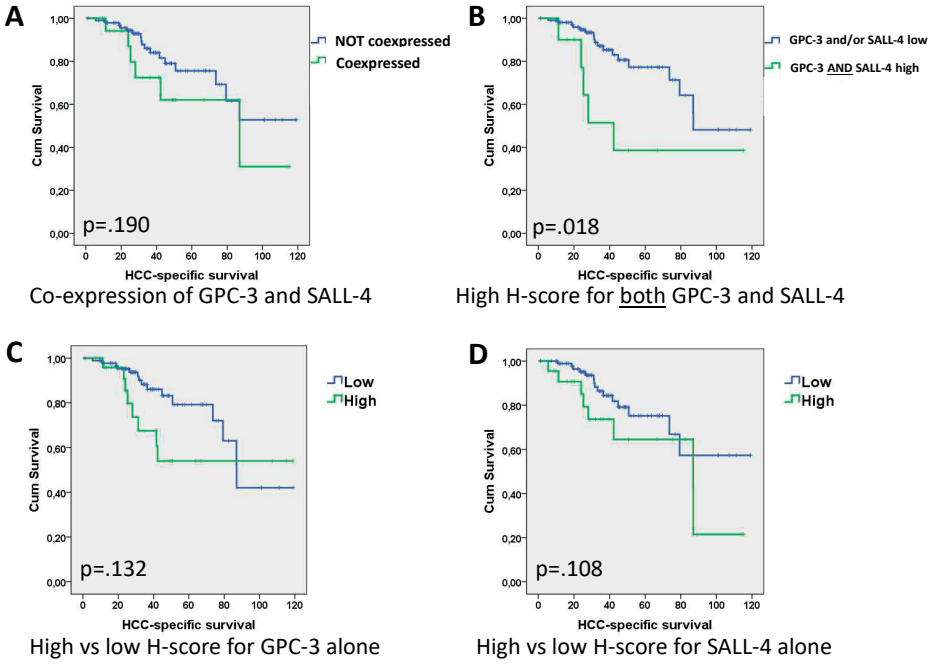
Supplementary Figure 1. Representative stainings for tumor tissue, TFL tissue, negative control and positive control for all antigens. The positive control tissues are: Testis tissue for the testis antigens (MAGE-A1, MAGE-A3/4, MAGE-A10, NYESO-1, SP-17 and SSX-2), liver cancer tissue for MDK, renal glomeruli for WT-1, stomach tissue for Survivin and MUC-1, and placenta tissue for AFP.



Supplementary Figure 1 (continued). Representative stainings for tumor tissue, TFL tissue, negative control and positive control for all antigens. The positive control tissues are: Testis tissue for the testis antigens (MAGE-A1, MAGE-A3/4, MAGE-A10, NYESO-1, SP-17 and SSX-2), liver cancer tissue for MDK, renal glomeruli for WT-1, stomach tissue for Survivin and MUC-1, and placenta tissue for AFP.



Supplementary Figure 2. TAA index and HCC specific survival



Co-expression of GPC-3 and SALL-4

High H-score for both GPC-3 and SALL-4

High vs low H-score for GPC-3 alone

High vs low H-score for SALL-4 alone

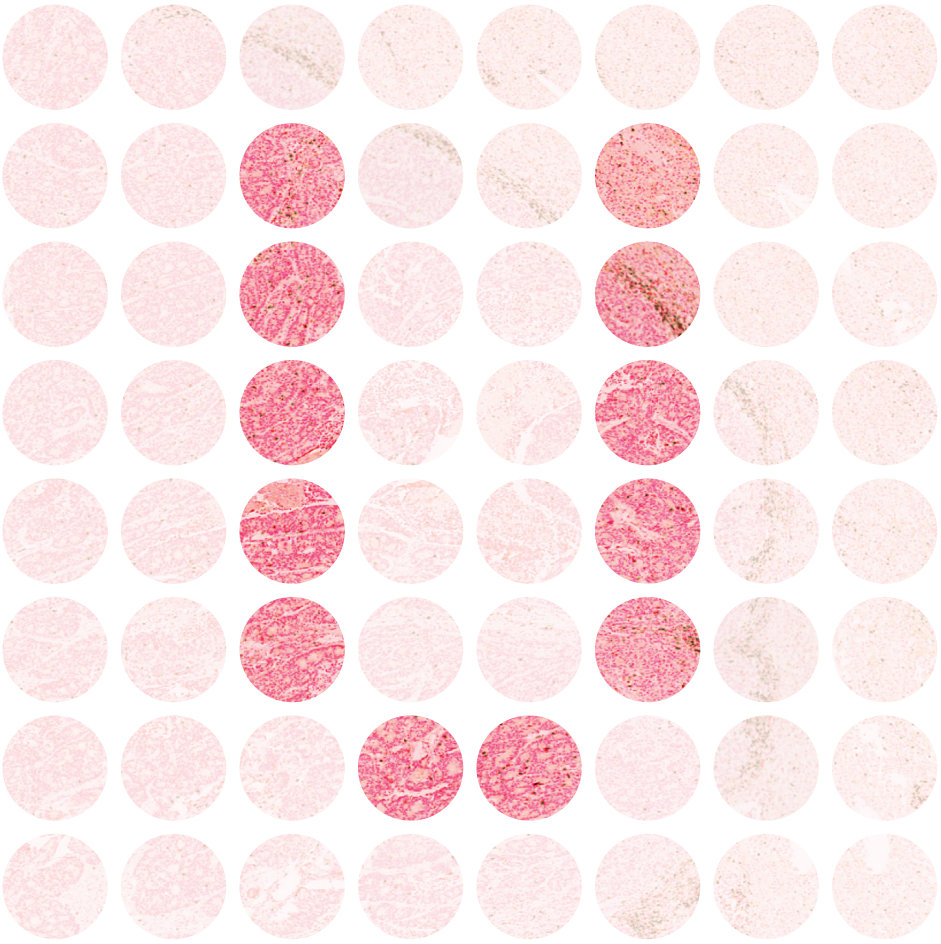
Supplementary Figure 3. Analysis of GPC-3 and SALL-4 co-expression for survival.

Supplementary Tables

Supplementary Table 1. Patient region of origin and hepatitis-B status

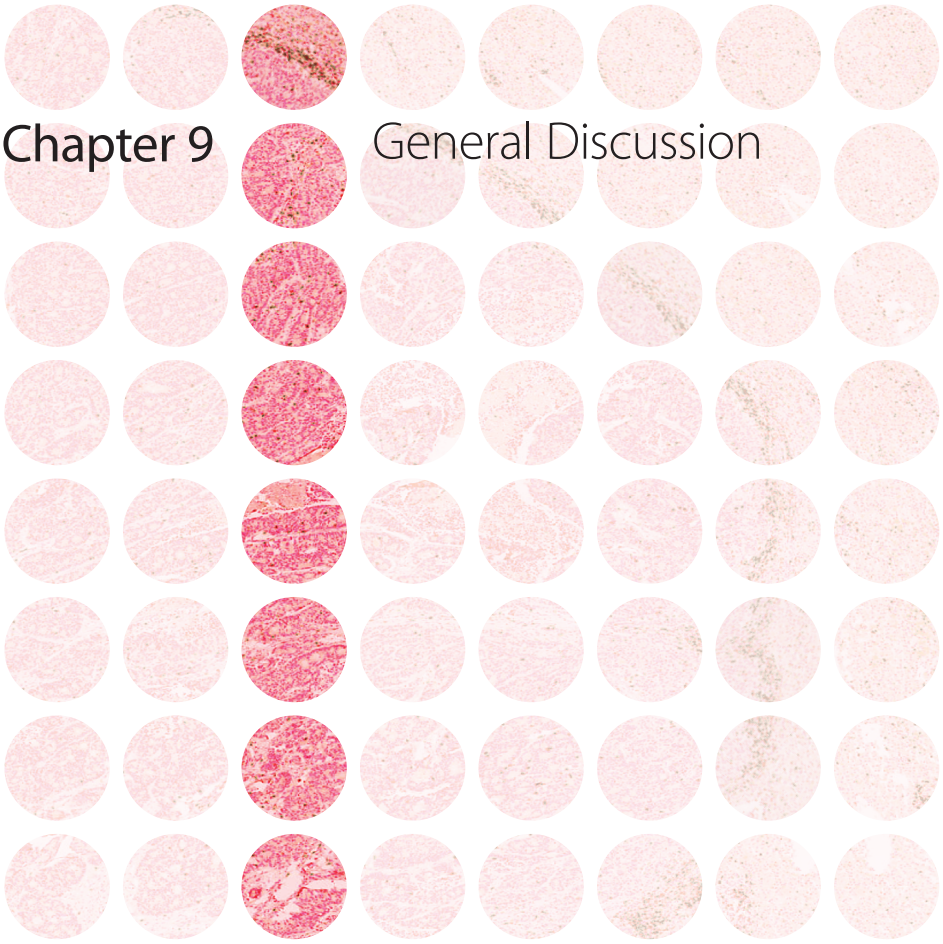
Region of origin	Frequency	Percent%	Hepatitis-B prevalence in our HCC cohort	Hepatitis-B population prevalence per CDC[1]
Western European	103	77.4	10/103 (9.7%)	**Low (<2%)**
Eastern European	3	2.3	0/3 (0%)	**Intermediate (2%-4%)**
Suriname	7	5.3	7/7 (100%)	
Middle Eastern	8	6.0	4/8 (50%)	
Sub-Sahara African	3	2.3	1/3 (33%)	**High (>5%)**
South-East Asian	9	6.8	8/9 (89%)	

[1]Ott JJ, Stevens GA, Groeger J, Wiersma ST. Global epidemiology of hepatitis B virus infection: new estimates of age-specific seroprevalence and endemicity. Vaccine. 2012.30(12):2212–9.



PART V

GENERAL DISCUSSION AND SUMMARY



Chapter 9 General Discussion

GENERAL DISCUSSION

Interest in the immune system's relationship to cancer has skyrocketed ever since treatments specifically aimed at manipulation of the immune system have entered clinical practice. The most successful of these treatments, namely the immune checkpoint inhibitors, are producing impressive results in several cancers (1-5). However, biomarkers that predict their efficacy are lacking. The current thesis is primarily concerned with examining the role of multiple different molecules as immune specific biomarkers in gastrointestinal cancers. To better understand the potential role of these biomarkers we have examined their protein expression in tumor tissues in Hepatocellular Carcinoma (HCC), Pancreatic cancer and Ampullary cancer, using tissue microarrays (Chapters 3, 4), as well as measured the circulating levels of two of these molecules in HCC (Chapter 7). In addition, we have investigated the prognostic value of Tumor Infiltrating Lymphocytes (TIL) in Colorectal Cancer Liver Metastasis (CRCLM) using full slides, enabling us to get a more global view of the tumor microenvironment (Chapter 5). Finally, in the process, we have studied expression of an important tumor antigen panel as potential vaccination targets in HCC (Chapter 8). However, several points regarding this thesis need to be reflected upon further.

Tumor infiltrating lymphocytes: importance of location and quality of infiltrate

Lymphocytic infiltration of tumors was first noted by Virchow in 1863 (6). However, it was only recently that tumor tissue from large cohorts of patients with cancer has been systematically examined for TILs. Some of the first breakthroughs came, interestingly, in primary colorectal cancer, where several large cohorts of patients showed that the more TILs present at the tumor site the better the survival of patients with colorectal cancer is (7-10). This same observation has been made in several other cancers (11). However, generic lymphocytic infiltration, while consistently prognostic, has not yet made it to clinical practice as a clinically useful biomarker. The reasons are several. First, TIL location is important. In a study by Galon et.al. (12), for example, it was shown that it is primarily the TILs at the invasive end of the tumor that are prognostically important. This may make sense, since it is at the invasive front of the tumor (the side where the tumor invades the bowel wall) that perhaps the immune system is putting up most of the fight against the cancer. Another important factor is the types of cells that constitute TILs. Simply looking at the generic amount of the lymphocytic infiltrate is not enough to produce clinically useful biomarkers. More specificity may come from looking at the quality, rather than the quantity, of the infiltrate such as the relative ratio of effector to regulatory cells. Both of these important factors, location and quality, need to be systematically addressed in all future studies concerning TILs in cancer.

In this thesis we consistently show that TILs are important in the cancer types we studied. We found that high $CD8^+$ TILs are associated with better cancer survival in HCC (Chapter 3) and high $CD8^+$/$FoxP3^+$ TIL ratio is associated with better cancer survival in pancreatic, ampullary and colorectal cancers (Chapters 4-5). Regarding the issue of location, some special considerations should be discussed regarding our observations. TILs were studied using three 0.6mm TMA cores in HCC and five 1mm TMA cores in pancreatic and ampullary cancers. TMA cores capture, by design, the intratumoral environment of a tumor. Thus, our results for HCC, pancreas and ampullary cancers cannot be generalized to lymphocytes present outside the tumors. But what about the difference between TILs at the invasive front and the center of the tumor, as has been distinguished for colorectal cancer in the study by Galon et.al. (12)? Unlike primary colorectal cancer, where an invasive front can be anatomically identified, such invasive fronts are not systematically present in HCC, pancreatic and ampullary cancers, or CRCLMs. HCC and CRCLM lesions are generally symmetrical and don't have a "leading edge". Pancreatic and ampullary cancers are associated with large amounts of fibrosis and it is frequently challenging to identify viable cancer cells, let alone distinguish an invasive front. However, in the design of our studies, we have avoided including necrotic areas and have, therefore, collected TMA cores from areas with viable cancer cells that are as close to the edge of the tumor as possible, without risking cutting through the peri-tumoral areas. It is unlikely to be able to improve the issue of "tumor location" further in HCC, pancreas and ampullary cancers and CRCLM.

To further understand the issue of tumor location we used full slides to study CRCLM. The fact that we show that it is the intra-tumoral TILs, and not the peri-tumoral TILs, that are prognostically significant makes sense and provides evidence to strengthen the hypothesis that it is not the amount of lymphocytes that are attracted to the tumor that are important but the lymphocytes that are able to enter the tumor itself that make the difference. It can also explain why studies in CRCLM, using TMAs, have reached similar conclusions to our study (13). TMA cores represent the areas in the tumor microenvironment that are most important; namely the intra-tumoral area.

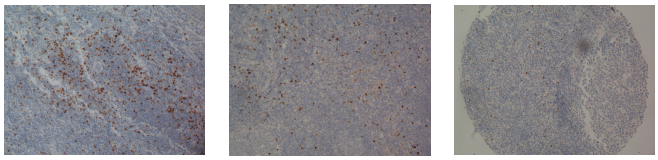
Regarding the quality of the infiltrate, we have studied both the presence and proportion of effector lymphocytes ($CD8^+$) and regulatory lymphocytes ($FoxP3^+$). Many studies have failed to do so, focusing either on the effector side (7-9, 14), or never comparing the relative presence of effector to regulatory lymphocytes (10, 15-17). In fact, we show both in pancreatic and ampullary cancers, as well as in CRCLM, that it is the ratio of effector to regulatory cells, and not the individual cell types, that are the most prognostic (Chapters 4 and 5). A large immune infiltrate may not be helpful if a large percentage of Treg cells are present. A smaller amount of effector T-cells, in the relative absence of Treg cells,

may be more effective. In fact our lab has shown convincingly that this proportion is important *in vitro* (18). It is of interest, however, that we were not able to study the presence of FoxP3$^+$ cells in HCC. FoxP3 staining produced too few positive cells in out TMA cores (Figure 1). In fact, only 4% of cases were found to have > 5 FoxP3$^+$ cells per core and in the vast majority of cases no FoxP3 positive cells were seen. However, FoxP3$^+$ cells were clearly identified in the peri-tumoral infiltrate and in the positive control tissues (Figure 1). Two reasons may account for this observation. First, FoxP3$^+$ cells are not very abundant in HCC tumors. Second, larger cores are required to study FoxP3$^+$ cells in HCC. We had no trouble of studying FoxP3$^+$ cells, for example, in pancreatic and ampullary cancer where five 1mm cores were used (the equivalent of 16.5 high powered fields). In future immunohistochemical HCC studies this issue should be taken into consideration.

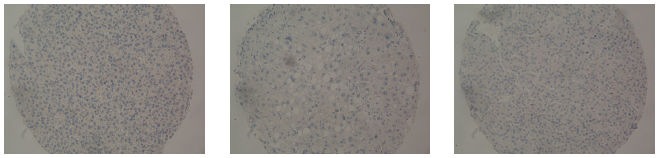
Differences with prior studies: attention to antibody quality

Given that immune checkpoint inhibitors targeting the PD-1 and PD-L1 axis have already been approved (1-5) it is of no surprise that issues regarding PD-L1 generate the most attention. Thus, when in both our studies in HCC (Chapter 3) and pancreatic and ampullary cancers (Chapter 4) we found results opposite to previously published literature there was added interest to properly and thoroughly validate our results. Specifically, while we found that low PD-L1 is associated with poor prognosis, prior studies in both HCC (19) and pancreatic cancer (20) had suggested the opposite. One of our main arguments regarding the differences in results rests on differences in the antibody clone used. While at first this may sound a somewhat simplistic argument, when closely looking at the evidence the issue of antibody validation becomes extremely important.

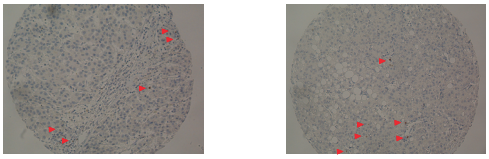
A popular PD-L1 antibody clone used for immunohistochemistry in the past was MIH1. In fact, all prior studies in HCC (19) and pancreatic cancer (20) have used this specific antibody clone. However, in two prior studies, the specificity of this clone for PD-L1 immunohistochemistry on formalin-fixed paraffin-embedded (FFPE) tissues has been questioned. For example, in the study by Valcheti et.al. (21), the authors tested 4 different PD-L1 antibodies for immunohistochemistry including the MIH1 clone. They found that the MIH1 clone failed to show the proper expected staining pattern in placenta tissue (that is staining in the trophoblastic cells of human placenta but absence of staining in the mesenchymal stromal cells and vessels of the chorionic villi). In the study by Gadiot et.al. (22), where again multiple PD-L1 antibodies were tested for immunohistochemistry, the MIH1 clone failed to properly stain human tonsil tissue. Therefore, eBioscience does not recommend this anti-PDL1 antibody for immunohistochemistry on FFPE tissues.



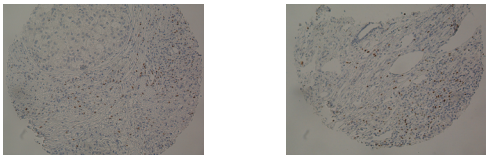
Various FoxP3 positive control tonsil tissues showing strong FoxP3 staining in selected cells



Typical tumor cores with complete absence of FoxP3 staining.
This patern accounted for the vast majority of tumor cores.



Tumor cores with 5 or more FoxP3 positive cells. These cores accounted for less than 4% of cases



Tumor cores capturing peritumoral infiltrate. These cores indicate that FoxP3 + cells are likely located in the periphery of the tumor and thus not captured by the vast majority of TMA cores.

Figure 1.

To further ensure that this was the case we did two things. First we stained human FFPE placenta tissue and human FFPE tonsil tissue with the MIH1 antibody and compared this to the staining of the validated antibody we have used (clone 405.9A11). We found that, while the 405.9A11 clone selectively stains trophoblastic cells in human placenta tissue and cells around the crypts of tonsil tissue, similar to what has been shown by Lyford-Pike et al (23), the MIH1 antibody, in agreement with the studies by Valcheti et.al., and Gadiot et.al., did not stain these control tissues properly (photographs shown in Chapter

3, Supplementary Figure 5). Second, we used the MIH1 antibody to stain HCC TMA cores, and compare the results to our stainings of the TMA cores with the 405.9A11 clone. In order to compare the two antibody clones, we first focused on tumor cores that did not express PD-L1 upon staining with the 405.9A11 clone. From the 6 cases that showed no staining with the 405.9A11 clone all but one showed considerable staining with the MIH1 clone (Chapter 3, Supplementary Figure 5). In addition, all other cases that showed any level of staining with the 405.9A11 clone, also showed staining with the MIH1 clone (pictures not shown).

The fact that our negative cases are mis-characterized as positive by the MIH1 clone can by itself explain the differences between ours and the prior studies. The MIH1 clone lacks specificity for PD-L1 in FFPE tissues and the observation that the cases with lack of PD-L1 staining have the worse prognosis cannot be made using the MIH1 clone. Issues of antibody specificity and validation are of the outmost importance for developing future immune biomarkers against cancer.

A similar situation exists regarding antibodies used in prior studies for HLA-G. The three prior studies on HLA-G expression in pancreatic cancer have issues with antibody quality. One study has used clone 4H84 (24), which has been shown to clearly be non-specific for HLA-G in multiple studies (25, 26), another study used a polyclonal antibody (27) and a third study did not provide details on the antibody used (28). In addition, none of the above studies provided photographs with positive control stainings for their antibodies. Issues of study quality could explain differences in results between different studies.

Intratumoral expression of immune inhibitory molecules: adaptive immune resistance at work?

There is a general agreement in our findings that lack of expression, or low expression, of immune inhibitory molecules is associated with poor cancer survival. This was shown for PD-L1, Gal-9 and HVEM in HCC (Chapter 3) and for PD-L1, Gal-9, HVEM and HLA-G in pancreatic and ampulary cancers (Chapter 4). The same observation has been made in several other cancers. For example, in the case of PD-L1, low expression of PD-L1 was found to be associated with poor survival in melanoma (29), gastrointestinal-stromal tumors (30), colorectal cancer (31) and non-small cell lung cancer (21). The above results are consistent with the adaptive immune resistance hypothesis that states that expression of immune inhibitory molecules is secondary to an active immune system and thus signifies an effective anti-tumor attack (29). Further supporting this hypothesis, in various cancers, are observations of PD-L1 overexpression in response to immune infiltrate (29) and IFN-γ (32, 33), as well as overexpression of PD-L1, IDO and T-regulatory cells in response to CD8 T-cell infiltration (34). Looking closer in our studies, the positive correla-

tion between PD-L1 and numbers of CD8+ TILs in both HCC (Chapter 3) and pancreatic and ampulary cancers (Chapter 4) is consistent with the hypothesis that enhanced expression of immune inhibitory molecules by tumor cells may reflect adaptive immune resistance.

Beyond adaptive immune resistance: other hypotheses

However, this may be only half the story. After all, immune inhibitory molecules suppose to "inhibit" the immune system, and not always signify a positive association to survival. In fact, several studies, in various cancers, have shown opposite results. For example, poor survival has been associated with over-expression of PD-L1 in melanoma (35), colorectal cancer (36) and renal cell cancer (37). It is known that cancers can induce an immunosuppressive tumor microenvironment by recruitment of immunosuppressive cells such as T-regulatory cells (Tregs), Myeloid Derived Suppressor Cells and Tumor Associated Macrophages and secretion of anti-inflammatory cytokines, such as TGF-β and IL-10. In addition, cancers can express a variety of immune inhibitory ligands, such as the molecules under study in the current thesis, but also others. Together, this immunosuppressive tumor microenvironment leads to the inactivation of the host anti-tumor immuno-surveillance system, as well as reduction of immunogenicity of cancer cells, leading to immune privilege of cancer cells. These factors may potentially explain findings linking high expression of PD-L1, and other immunosuppressive molecules, to poor prognosis.

Yet, additional studies, show no prognostic significance of PD-L1 in various cancers (22, 38-40). Indeed, especially regarding PD-L1, there is a current controversy: "how can expression of PD-L1, an immunosuppressive molecule, be associated with both a good and a poor cancer prognosis, over several studies and cancer types"? While differences in technique, such as validity of the antibody clone used, measuring expression specifically in cancer cells versus the tumor micro-environment, as well as differences in patient cohorts (cancer type, early vs late stage of disease, completeness of patient follow up) may explain some of the differences, it likely does not explain everything. Instead, it is likely that the relationship of tumor microenvironment immune biomarkers and prognosis is not linear. Other immunologic factors come into play.

The answer may be that looking at individual immune inhibitory molecules, one at a time, may be too narrow. The wider context needs to be taken into consideration, before the prognostic role of these molecules can be discerned. For example PD-L1 is likely best viewed in the context of the immune infiltrate, and not by itself. Expression of PD-L1 in the context of an active immune infiltrate is likely to indicate adaptive immune resistance (PD-L1 expressed by cancer cells in response to the immune infiltrate), as has

been demonstrated before in melanoma (29) and recently in HCC (41). On the other hand, the absence of PD-L1 in a setting of an immune infiltrate could indicate immune tolerance to the tumor or the presence of molecular suppressors of PD-L1. Similarly, absence of PD-L1 staining in the context of an absent, or inactive, immune infiltrate is likely to indicate immune ignorance (the immune system is "unaware" of the cancer). Finally, high expression of PD-L1 in the absence of an immune infiltrate can signify intrinsic induction of PD-L1, secondary, for example, to chromosomal amplification (42) (and thus unrelated to an immune infiltrate). Such a contextual model for PD-L1 expression has already been suggested by Teng MW et.al (43). Since the findings in this thesis can only be partially explained by the adaptive immune resistance hypothesis, other factors are likely playing an important role on a patient to patient basis.

A similar argument can be made by looking at the presence of Tregs. Despite the fact that Tregs are clearly immunosuppressive, their presence does not necessarily, by itself, signify a poor prognosis. In our study in pancreas and ambulatory cancers (Chapter 4) elevated numbers of $FoxP3^+$ cells were associated with good prognosis. This has been shown before, as for example in the large study (967 stage II and stage III colorectal cancers) by Salama et. al., (10). It is likely that the number of $FoxP3^+$ cells is more important in the context of the total immune infiltrate (you need an immune infiltrate to have $FoxP3^+$ cells after all). So both a very high absolute number of $FoxP3^+$ cells (dominating and suppressing the immune infiltrate), as well as a very low absolute number of $FoxP3^+$ cells (associated with a small immune infiltrate, indicating reduced immunogenicity and absence of immune surveillance) may be associated with poor prognosis.

Basically, the prognostic role of immune-regulatory molecules, such as PD-L1, or the prognostic role of the immune infiltrate, can be influenced by the relative proportions of multiple tumor microenvironment factors, which may in turn depend on the cancer subtype, the cancer stage (very early, vs advanced, vs metastatic), and/or prior treatments. This could account for discrepancies between studies. But also it could account for the inability of individual immune molecules to serve as important biomarkers. In fact, our approach to examine the prognosticating role of PD-L1 in HCC, pancreatic and ampullary cancers, in the context of the quality of the immune infiltrate ($CD8^+$ and $FoxP3^+$ cells), as well as other immune molecules (Gal-9, HVEM, HLA-G), is justified considering the above. We show that combining multiple individual potential biomarkers can lead to more useful cancer immune biomarkers (Chapters 3 and 4).

At this point it is important to bring into focus our findings on the Tumor Associated Antigen Index (TAA index) in HCC (Chapter 8). TAA index is a direct representation of the number of TAA expressed by a given cancer. A high TAA index (more TAA expressed) has

previously been found to be associated with a better survival in HCC (44). We found similar findings using a broader panel of TAAs in HCC (Chapter 8). We showed that patients who express 7 or more TAAs have a significantly better prognosis than patients who expressed 2 or fewer TAAs (Chapter 8, Supplementary Figure 2). One may hypothesize that patients who express few TAAs exhibit a form of immune ignorance. If that is true then one would expect tumors with fewer expressed TAAs to have fewer TILs and tumors with high TAA index to have more TILs. In the subsequent unpublished table from our study, we see that indeed there is a relationship between $CD8^+$ TIL and TAA index in HCC in the direction we hypothesize.

Crosstabs analysis of $CD8^+$ cells in relation to TAA index in HCC

Mantel-Haenszel test of trend **p=.048**		**CD8+ TILs/core**			
TAA index		<100	>100	%	Total
	0-2	7	0	0%	7
	3-6	93	13	12%	106
	7-9	10	4	29%	14
	Total	110	17		127

Tempting as it may be, however, it should be noted that causation cannot be established from this association. It is unclear if the expressed TAAs are due to the presence of TILs or vice-versa. The regulation of expression and immunogenicity of many TAAs, such as the testis antigens, have not been well studied at the moment. Thus, while it is possible that TAA expression is also related to the phenomena discussed above, one needs to wait until more is known on the function of many of the molecules involved before specific inferences can be made. One first step, for example, would be to examine if HCC cell lines express testis tumor antigens when exposed to T-cell derived cytokines such as IFN-γ, or are co-cultured with tumor isolated effector T-cells.

Beyond PD-L1: The argument for using multiple biomarker panels

To date the only immune biomarker available for prediction of effect of immunotherapeutic agents is tumor tissue immunohistochemical evaluation of PD-L1 (45, 46). While the current thesis focuses on the development of prognostic biomarkers, these same biomarkers could in the future become predictive biomarkers to different immunotherapeutic strategies. In addition, the pitfalls regarding the use of PD-L1 IHC as a predicative biomarker are instructive and can be generalized to other settings. There are several reasons that PD-L1 IHC, by itself, is far from an ideal biomarker at the moment. We will focus on differences between antibody clones, the differences between membranous and cytoplasmatic staining and the poor predictive biomarker ability of these assay.

The currently available diagnostic immunohistochemistry assays, generally, accompany treatment with immune checkpoint inhibitors targeting the PD-1/PD-L1 axis. Higher expression of PD-L1 is associated, in all cases, with improved response to these immune checkpoint inhibitors. However, different antibodies are used in companion diagnostic assays for immunohistochemical evaluation of tumor PD-L1 expression delivered by pharmaceutical companies with different PD-L1/PD-1 immune checkpoint inhibitors (47). Treatment with Nivolumab is accompanied with a companion kit using PD-L1 antibody clone 28-8, treatment with Pembrolizumab with clone 22C3, treatment with Atezolizumab with clone SP142 and treatment with Durvalumab with clone SP263. The reason for the difference is that the companies developing and testing the individual drugs also developed their own companion biomarker tests. Going beyond these companion tests there are several other validated PD-L1 clones used in non-treatment IHC studies such as E1L3N, 5H1 and 9A11 (the clone used for our studies). It is of interest that many of the above PD-L1 clones bind the extracellular domain of PD-L1, while others (SP142, E1L3N and 9A11) bind the intracellular domain. As a result, differences in results are possible when using so many different types of assays to measure the same molecule. In fact, each tests has, during the biomarker optimization process, acquired a different threshold for what constitutes positive PD-L1 expression, ranging from 1% to 50% positive membranous staining (47).

In addition, all the above assays measure membranous expression of PD-L1. However, both membranous and cytoplasmatic staining have been described, for PD-L1, in various cancers before (29, 48-50). In fact, in HCC, pancreatic and ampullary cancers we only observed cytoplasmatic staining. This is despite the fact that the antibody we used for immunohistochemistry, namely clone 9A11, was developed specifically to bind the intracellular domain of PD-L1, in hopes that this would enhance the membranous staining pattern (51). Nevertheless, cytoplasmatic staining has been generally ignored. It is thought that cytoplasmatic staining is poorly reproducible between studies and simply interferes with the evaluation of membranous staining. However, that does not mean that cytoplasmic staining is of no biologic significance. One hypothesis if that cytoplasmatic staining represents intracellular stores of PD-L1 that are transported to the cellular membrane (29), or secreted as a functional soluble molecule (52), upon contact with or effector TILs or the presence of IFN-γ. In fact, transport of cytoplasmatic stores to the membrane, upon activation, has been demonstrated for another important immune inhibitory molecule, namely CTLA-4 (53, 54). It is not known yet if that is the case with PD-L1. Regardless, the fact that we consistently found, in 3 different cancers, that cytoplasmatic expression of PD-L1 is prognostic for survival makes a strong case for not ignoring cytoplasmatic PD-L1 expression, although standardization may require computer assisted imaging technology.

Finally, unlike biomarkers such as EGFR, which represent the status of driver oncogenes, blocking the PD-1/PD-L1 axis is only one of several immune inhibiting mechanisms available to cancer. While patients without an activating EGFR mutation have no benefit, or even harm, from anti-EGFR therapy in lung cancer (55), patients with a negative PD-L1 test have a 6% to 41% chance to respond to treatment targeting the PD-1/PD-L1 axis (56). For the development of ideal predictive biomarkers this should be reduced to near 0%.

Optimization of immunohistochemical detection of PD-L1 as a single biomarker, given the above issues, is highly unlikely. In addition, PD-L1 cannot represent the complete immunologic status of the tumor microenvironment. Thus, looking at multiple biomarkers simultaneously, such as PD-L1 expression in the context of the immune infiltrate, or other immune inhibitory molecules such as Galectin-9, HVEM or HLA-G is inevitable, if ideal immune biomarkers are to be developed.

Circulating immune biomarkers: a story of their own?

Cancer is known to be associated with systemic inflammation since the 19th century (6). It is unclear, however, if systemic inflammation is good or bad for the patient. Multiple observations of spontaneous regression of cancers following immunestimulation, such as fever and infections, indicate a positive role of inflammation in fighting cancer (57). However, the fact that cancer progression is frequently associated with clinical signs of inflammation, in addition to the well known causative role of chronic inflammation in cancer, indicate an averse role (58).

Previously we discussed how the quality of the immune infiltrate, at the tumor microenvironment, is more important that the quantity of the immune infiltrate, regarding prognosis. Could a similar hypothesis be supported regarding systemic inflammation? In Chapter 6 we discuss how various inflammatory indices are associated with prognosis in various cancers. It is known that non-specific measures of inflammation, such as the c-reactive protein, is associated with poor prognosis in various cancers (59). This may be due to the fact that systemic inflammation is a consequence of cancer, such as recognition of tumor antigens by immune cells in a growing cancer. Since the inflammation is not effective against the cancer, elevated c-reactive protein or elevated neutrophil count are not protective against the cancer. However, an equally interesting observation is that low absolute lymphocyte count (ALC), is also associated with poor survival in various cancers (60). In fact, the strongest systemic prognostic biomarkers in cancer are ratios of inflammatory markers such as the neutrophil to lymphocyte ratio (NLR) (61). Here, the lymphocytes represent the effective side of the immune system while neutrophils represent non-specific, ineffective, inflammation. So far these immune markers represent laboratory tests available to all clinicians, a remarkable fact in its own right. However,

one may go deeper and examine different lymphocytic subsets. A high proportion of circulating T-regulatory cells are associated with poor prognosis in various cancers (62-64). Thus not all lymphocytes are good, but it depends on the particular dominating subtype. This means that just like inflammation in the tumor microenvironment, it is the quality and not the quantity of systemic inflammation that is more important in determining prognosis, or even perhaps the ability of patients to respond to the various new immunotherapeutic strategies.

Regarding our cohorts we also examined the NLR as a possible prognostic marker, although the results are not published yet. In pancreatic and ampullary cancers a high NLR, before resection, is associated with worse cancer specific survival after resection (HR 1.89, 95%CI 1.08-3.33, p=.027). In patients with resected HCC a high NLR is associated with a trend towards worse HCC specific survival (HR 2.64, 95%CI 0.84-8.36, p=.098). This indicates that at least the NLR should be more carefully examined as a clinical biomarker in these diseases.

In Chapter 7 we examine the prognostic role of circulating forms of immune inhibitory molecules in HCC patients undergoing tumor resection or liver transplantation and compare their circulating levels to intratumoral expression of the same molecules, a novel idea. From all the immune inhibitory molecules studied in the current thesis only PD-L1 and Galectin-9 had quality Elisa kits available at the time. IDO metabolites have also previously been studied by HPLC but the expense of measuring these metabolites is significant. We were surprised to find out that circulating PD-L1 and Galectin-9 were not reflective of either the intratumoral expression or the TFL expression of these molecules. In addition, the circulating levels of PD-L1 and Galectine-9 provide independent prognostic information compared to their respective intra-tumoral expression. Finally, circulating levels could be added to intratumoral levels to improve prognostication. In fact, patients with both high circulating and intra-tumoral PD-L1 or Gal-9 rarely ever died from cancer, indicating a complementary relationship for these immune biomarkers. All this implies that the source and pathophysiologic role of circulating PD-L1 and Galectine-9 is different than their respective source and pathophysiologic role in the tumor microenvironment.

Regarding the source, while tumor cells express immune inhibitory molecules (as we have shown confidently in this thesis), other cells, such as various types of immune cells, and normal cells such as hepatocytes, are also known to express these molecules. Release of PD-L1 into the circulation has been shown to occur by various tumor cell lines (52), mature dendritic cells (65) and by circulating macrophages (66, 67). Regarding Galectine-9, while cancer cells are known to secrete this molecule by exosomes (68), the

fact that elevated levels have been found in various benign inflammatory liver diseases indicates additional sources (69-71). The pathophysiologic role of these molecules in the circulation is also poorly studied. However, it is known that the circulating form of PD-L1 retains its PD-1 binding domain and immunosuppressive properties (52). Since PD-L1 and Galectine-9 are known to be expressed in tumor cells and immune cells in response to inflammation (i.e. IFN-γ), it would be interesting to study if PD-L1 is also released into the circulation in response to effective, anti-tumor, inflammation. If so, it is likely that the presence of these molecules in the circulation is, at least partly, a reflection of adaptive immune resistance.

Tumor associated antigen expression in HCC

In Chapter 8 we do not look at the expression of immune inhibitory molecules or TILs in cancer but at the expression of Tumor Associated Antigens (TAAs). TAAs are important components for a different type of immunotherapy, namely cancer vaccination. While cancer vaccination is lagging behind in overall success in comparison to the immune checkpoint inhibitors, it was nevertheless the first immunotherapy to be approved against cancer (72, 73) and there are currently hundreds of cancer vaccination clinical trials underway. It is very likely that in the future cancer vaccination will play an important role in the fight against cancer. Thus identification of TAAs is of the outmost importance. In Chapter 8 we show that Annexin-A2, Glypican-3, MAGE-C2 and MAGE-C1 is a panel of TAAs that combine the highest prevalence of expression in tumor tissues with a lack of expression (or in case of Annexin-A2 limited expression) in TFL tissue. This makes them potentially good candidates to include in tumor vaccination studies against HCC since they meet important criteria of what constitutes ideal tumor antigens (74). We show that in 95% of patients at least one of these antigens is expressed while in 47% of patients two or more of these antigens are expressed.

Given that single peptide vaccines have been generally unsuccessful in treating cancer, to date the focus of the international research community is to develop new vaccine strategies. One of these novel techniques is vaccination using the messenger mRNA of the corresponding tumor antigen. Upon injection, RNA is taken up by dendritic cells and translated into proteins, which are degraded into peptides and presented to both $CD4^+$ and $CD8^+$ T cells. Initial trials have already established safety as well as the generation of appropriate T-cell responses (75).

To further develop our findings from Chapter 8, our research group has acquired mRNA from Glypican-3 and MAGE-C2. The mRNA is transferred to antigen presenting cells by electroporation and the ability of these cells to stimulate appropriate T-cell responses,

in vitro, is evaluated. The next step is to treat patients with HCC, who express these antigens, with mRNA based vaccination.

TMAs versus full slides

Given the fact that cancer is extremely complex the question arises: are TMAs suitable for studying cancer? To properly answer this question one must consider what the specific research question under consideration is. TMAs allow a significant reduction in the workload and cost of immunohistochemistry, allow better control of the experimental conditions used (since multiple tissues are present on one microscopic glass slide) and lead to sparing significant amounts of tumor tissue (76). For example, the tissue blocks we used for constructing our TMAs for Chapters 3 and 4 can still be used by us, or other investigators, to study additional research questions. Regarding the criticism that focal expression of biomarkers can be missed by small TMA cores it should be noted that a strong correlation between the results of TMA and full slides has been demonstrated in several studies (77-82). In fact, in Chapter 4 of this thesis we show that there was no difference in staining evaluation between TMA cores and full slides for selected cases (Supplementary Figure 4, Chapter 4). The correlation between TMAs and full slides is true even for biomarkers that are known to be focally expressed (76). The correlations in the case of focally expressed biomarkers, however, is inversely proportional to the size of the study cohort. Thus TMAs are suitable when large cohorts of patients are studied.

However, TMAs are not suitable for answering questions of complex spatial relationships in the tumor microenvironment. In Chapter 5, where both the intra-tumoral and the peri-tumoral composition of the immune infiltrate was studied, in a small cohort of patients with CRCLM, the use of TMAs would have been inappropriate. However, if the results of our Chapter 5 are validated, namely that it is the intra-tumoral composition of the immune infiltrate that is most important, then one may return to TMAs to study larger cohorts of patients with CRCLM.

Moreover, in Chapter 4, we were not able to study the expression of immune inhibitory ligands in the surrounding non-tumoral tissue. Indeed we cut and stained 2 x 1mm cores from the surrounding "normal pancreas" area from each block. However, we now feel strongly that 2 x 1 mm cores are not enough to fully characterize the peri-tumoral area in pancreatic and ampullary cancer. This is because of the significant heterogeneity in histology we observed. The histology ranged from completely normal pancreatic tissue, to stromal tissue without invasive cancer cells, to areas of destructed "normal tissue" with significant atypia. Capturing the complexity of the peri-tumoral environment requires perhaps full slides or significant more TMA cores (from the non-tumorous areas). In fact, the molecules we examined are expressed, in several cases, by atypical, non-invasive

cells and occasionally by normal pancreas. However, a systematic determination of staining patterns could not be confidently made using 2 TMA cores, thus we do not discuss findings in stainings in the peri-tumoral areas. Proper future systematic study of the peri-tumoral microenvironment in pancreatic and ampullary cancers would require full tissue slides.

Expansion of biomarker panels

The molecules we examined in our thesis are not exhaustive. We focused on molecules with a well validated antibody and relatively understood biology. However, as knowledge on the interaction of the immune system with cancer expands, more molecules need to be tested. Regarding TIL markers one may focus on molecules beyond CD8 and FoxP3. For example, markers of cellular cytotoxicity, such as granzyme B (83, 84), myeloperoxidase (85), or NKT cell markers, such as CD1d or TCR Vα24-Jα18 (86, 87), may prove useful. In addition, the memory T-cell marker CD45RO has shown promise in the setting of colorectal cancer (88). Regarding immune inhibitory molecules other members of the B7 superfamily, which includes PD-L1 (B7-H1), such as B7-H3 (89) and B7-H4 (90, 91) deserve attention. At the moment a clear link between the immune system and galectins other than Galectin-9 has not been established. HLA-E, another non-classical HLA molecule (92), should also be tested.

Future directions: Clinical development of immune biomarker profiles

Several issues regarding future development of immune biomarkers have already been discussed: use of properly validated antibodies, appropriate use of TMAs, incorporation of cytoplasmatic staining when applicable, use of circulating molecules, expansion of the molecules under study and development of biomarker panels with multiple molecules. However, several steps are required before such an antibody panel reaches clinical development. Once information on promising potential biomarkers is established on TMAs, the potential biomarker panel will need to be validated on full slides. This is because decisions for individual patients are performed on full slides. Even if the issue of heterogeneity is minimized by the use of large cohorts in TMA research (76), heterogeneity is a significant issue when decisions on individual patients need to be made. Here again, the hope is that with the use of a biomarker panel heterogeneity in one molecule may be less detrimental than in a single biomarker situation. However, issues of heterogeneity and reproducibility will need to be resolved.

Of necessity will be the repetition of the stainings, for the chosen molecules, on a large well characterized cohort of patients with a given disease. At this step systematic work with an experienced pathologist(s), establishing diagnostic criteria, is required. Once confidence is build regarding staining evaluation, the results will need to be validated in

an independent external cohort, using the same antibodies and assays. Then a prognostic biomarker can be introduced to clinical care.

Of more importance than prognostic biomarkers are predictive biomarkers to immunotherapeutic strategies. Here, tissue from completed randomized controlled studies need to be accessed and the biomarker panel retested. Ideally, this step requires both (re) discovery and validation cohorts. The final goal is incorporation of the biomarker panel in a clinical trial, which requires significant standardization of the biomarker assay, in addition to other recommended criteria (93).

Selection of patients highly unlikely to benefit from immunotherapy, or patients highly likely to benefit from immunotherapy, will have an immediate obvious clinical benefit. Moreover, discovery of predictive immune biomarkers will allow future clinical trials to enrich their testing cohorts with patients more likely to benefit, thus speeding up overall research efforts. Finally, it is likely, that a well-developed immune biomarker panel, which characterizes the complexity of the tumor immune microenvironment to a reasonable extent, will not be specific to a given treatment but will extend its usefulness to include other types of immune checkpoint inhibitors, or even other immunotherapeutic strategies entirely.

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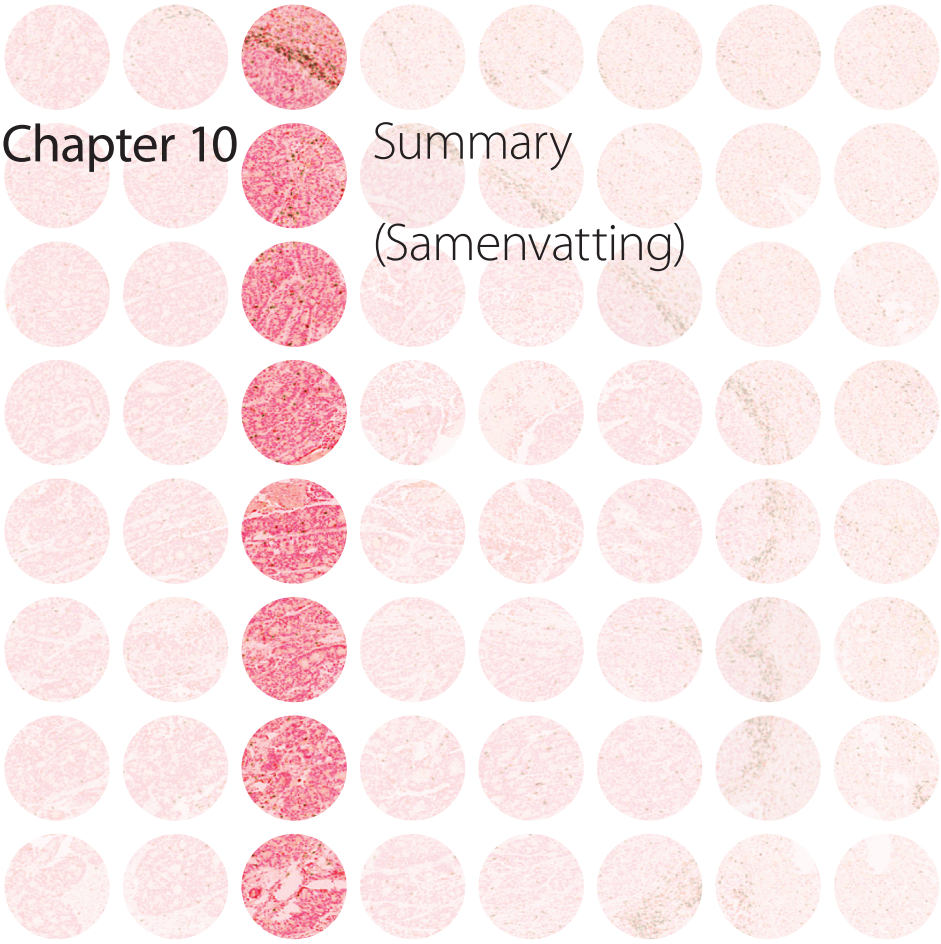


Chapter 10 Summary (Samenvatting)

ENGLISH SUMMARY

The first observation that immune cells can infiltrate tumors was made by Virchow in 1863. Ever since, it was suspected that the immune system plays an important role in cancer. However, it is only recently that treatments activating the immune system (immunotherapy) have been approved for treating patients with cancer such as melanoma and cancer of the prostate, lung, kidney and bladder. Even though these treatments are successful not all patients benefit and in fact the treatments can sometimes be toxic. Thus, finding biomarkers that predict the patients who benefit from immunotherapy is of the outmost importance.

In the current thesis we examine the role of several immune molecules as possible biomarkers in cancers of the gastrointestinal tract (hepatocellular carcinoma, pancreatic cancer, ampullary cancer and colon cancer). These molecules are frequently expressed by cancer cells to protect themselves from the immune system. We also examine the role of tumor infiltrating lymphocytes as possible biomarkers. In the process we also identify a panel of tumor antigens that can serve as potential targets for vaccination.

In chapter 1 we give a general introduction on the importance of the immune system in cancer and the rationale behind the work of this thesis. In chapter 2 we continue our introduction by focusing on immune inhibitory mechanisms, as well as experimental immunotherapies, in pancreatic cancer. In chapter 3 we discuss the role of several potential immune biomarkers in hepatocellular carcinoma. We show that a panel of markers, namely PD-L1, Galectin-9 as well as $CD8^+$ immune infiltrating cells, can be an important prognostic biomarker. Interestingly, the patients who express the immune inhibitory molecules PD-L1 and Galectine-9 survive longer than patients who do not express these molecules, the opposite of what one would expect. In chapter 4 we focus on cancers of the pancreas and the ampulla. We also show that the combination of PD-L1, Galectine-9 and other molecules, such as HVEM and HLA-G, in combination with the presence of immune infiltrating cells (CD8/FoxP3 ratio) can be an important biomarker in these cancers. We also show, again, that patients who express these molecules survivelonger than patients who do not express them.

Why is it that expression of molecules that protect cancer cells from the immune system are associated with better patient survival. In chapters 3 and 4, as well as in the general discussion in chapter 9, we discuss possible reasons. One of the most important reasons may be what is known as “adaptive immune resistance”. This means that cancers that express immune inhibitory molecules do so because they are under active attack from immune cells. These molecules are a sign that the immune system still recognizes and

attacks the cancer cells. When a cancer cell does not express these molecules any more it may be beyond the detection of the immune system and thus a bad sign for the patient.

In chapter 5 we examine the importance if immune infiltrating cells in liver metastasis from colorectal cancer. In this work we use larger tumor sections in order to understand if the location of the infiltrating immune cells is important. It is known that the majority of immune cells attracted to cancer stay around the tumor and not penetrate inside the tumor. We show that it is the immune cells that infiltrate into the cancer itself that are important for patients survival and not the cells that stay outside. We also show that it is not the amount of cells that penetrate inside the tumor but the relative proportion of effector immune cells that fight cancer ($CD8^+$) to the regulatory immune cells that help cancer ($FoxP3^+$). This last observation, namely that it is the quality rather than the quantity of the immune cells that is important in survival, we also made in chapter 4 about pancreatic and ampullary cancers.

In chapters 6 and 7 we move beyond cancer cell expression of biomarkers and discuss possible biomarkers that are present in the circulating blood. In chapter 6 we discuss what is known about routine laboratory tests of the immune system and their relationship to cancer survival. We discuss how routine laboratory tests, such as neutrophils, lymphocytes and platelets, or combinations of these markers, have the ability to predict which patients with cancer will live longer. In chapter 7 we investigate the role of PD-L1 and Galectin-9 in circulating blood of patients with hepatocellular carcinoma. We show the levels of these immune inhibitory molecules in the circulating blood also predict cancer survival, in a similar way as with their expression by cancer cells (chapter 3). However, the patients with high PD-L1 and Galectine-9 in the blood are not always the same as the patients with who express high PD-L1 and Galectine-9 in the cancer cells. Thus, information from the circulating blood is independent, to a certain extent, from information from the cancer site. Patients who have high PD-L1 or Galectine-9 in both the blood and the cancer site have a very good cancer prognosis, while patients who have low PD-L1 or Galectine-9 in both the blood and the cancer site have a very poor prognosis.

In chapter 8 we investigate tumor associated antigens that can become possible targets for vaccination in hepatocellular carcinoma. We show that a panel of four antigens, namely Annexin-A2, Glypican-3, MAGE-C1 and MAGE-C2, are expressed in a significant amount of patients, while at the same time they are expressed little (Annexin-A2) or not at all (Glypican-3, MAGE-C1 and MAGE-C2) in the surrounding normal liver. These characteristics make them possible good targets for vaccination and our lab is currently working on developing such a vaccination strategy in hepatocellular carcinoma. In addi-

tion, in chapter 8, we also show that patients who express lots of these tumor antigens (7 or more) do better that patients who express very few of these tumor antigens (2 or fewer). In the general discussion, in chapter 9, we hypothesize that perhaps, similarly to the expression of the immune inhibitory molecules, expression of too few tumor antigens could mean that the cancer cells are beyond detection of the immune system, explaining the poor prognosis of these patients.

In chapter 9 we provide a general discussion of our thesis. We focus on issues that require more discussion, such as providing additional hypothesis that explain our findings. We also discuss technical aspects of our thesis, such as the importance of antibody quality used for immunohistochemistry and the differences, and appropriateness, of using tissue microarrays versus full tissue slides. Finally, we also provide ideas on how to continue this research forward, with the goal of successfully developing clinically useful immune specific biomarkers for the era of cancer immunotherapy.

NEDERLANDSE SAMENVATTING

De eerste waarneming dat immuuncellen tumoren kunnen infiltreren is gedaan door Virchow in 1863. Sindsdien werd lang vermoed dat het immuunsysteem een belangrijke rol speelt bij kanker. Het is echter pas sinds kort dat behandelingen die het immuunsysteem activeren (immunotherapie) zijn goedgekeurd voor de behandeling van patiënten met kanker, zoals melanoom en prostaat-, long-, nier- en blaaskanker. Hoewel deze behandelingen succesvol zijn, hebben niet alle patiënten hier baat bij en zijn deze behandelingen soms zelfs toxisch. Derhalve is het van het grootste belang om biomarkers te vinden die kunnen voorspellen welke patiënten baat kunnen hebben bij immunotherapie, en welke niet.

In dit proefschrift onderzoeken we verschillende immuunmoleculen als potentiele biomarkers in gastrointestinale kankers (i.e. hepatocellulair carcinoom, pancreascarcinoom, papilcarcinoom en colorectaal carcinoom). Deze moleculen worden vaak tot expressie gebracht door kankercellen om zich te beschermen tegen het immuunsysteem. We onderzoeken ook de rol van tumor-infiltrerende lymfocyten (TIL) als mogelijke biomarker. Daarnaast identificeren we een panel van tumor antigenen die als potentiële componenten van toekomstige kanker vaccins kunnen dienen.

In hoofdstuk 1 geven we een algemene inleiding over het belang van het immuunsysteem bij kanker en leggen we de grondgedachte voor het werk van dit proefschrift. In hoofdstuk 2 gaan we door met de inleiding door te focussen op immuunremmende mechanismen, evenals experimentele immuuntherapie bij pancreascarcinoom. In hoofdstuk 3 onderzoeken we m.b.v. coupes van kleine stukjes tumorweefsel (zogenaamde weefsel microarrays) de rol van de verschillende potentiële immune biomarkers in hepatocellulair carcinoom. We laten zien dat immunohistochemische bepaling van een panel van immuunmoleculen, namelijk PD-L1, Galectine-9, op tumorcellen, en cytotoxischer $CD8^+$ TIL, een accurate prognostische biomarker is voor overleving na chirurgische resectie van HCC. Een interessante bevinding is dat de patiënten die de immuunremmende moleculen PD-L1 en Galectine-9 op hun tumorcellen tot expressie brengen langer overleven dan patiënten die deze moleculen niet tot expressie brengen. Dit is het tegenovergestelde van wat men zou verwachten.

In hoofdstuk 4 richten we ons op pancreascarcinoom en papilcarcinoom. We laten zien dat immunohistochemische bepaling van de expressie van een panel van PD-L1, Galectine-9, HVEM en HLA-G op tumorcellen in combinatie met de ratio van cytotoxische $CD8^+$ T cellen en regulatoire $FoxP3^+$ T cellen in TIL een accurate prognostische biomarker is voor overleving na chirurgische resectie van deze kankers. Opnieuw laten we zien dat

patiënten die deze moleculen tot expressie brengen langer overleven dan diegenen die dat niet doen. Hoe komt het dat de expressie van moleculen die de kankercellen tegen het immuunsysteem beschermen is geassocieerd met een betere overleving voor de patiënt? In hoofdstuk 3 en 4, maar ook in de algemene discussie in hoofdstuk 9 bespreken we mogelijke redenen. Een van de belangrijkste redenen is dat kankers die immuunremmende moleculen tot expressie brengen, dit doen omdat ze actief worden aangevallen door het immuunsysteem. Deze moleculen zijn een teken dat het immuunsysteem de kankercellen nog herkent en aanvalt. Als een kankercel deze moleculen niet meer tot expressie brengt, kan het betekenen dat deze niet meer wordt gedetecteerd door het immuunsysteem. En dit is een slecht voorteken voor de patiënt.

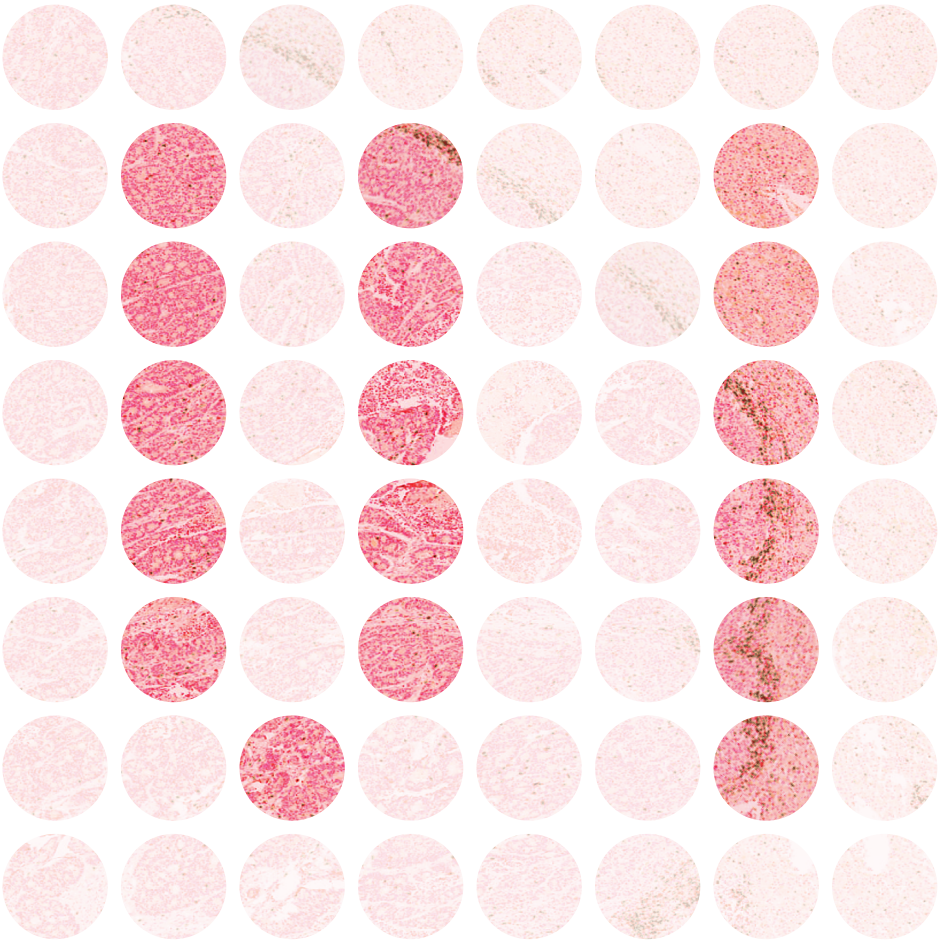
In hoofdstuk 5 gaan we in op het belang van TIL in levermetastases van patienten met een colorectaal carcinoom. In deze studie gebruiken wij grote tumorcoupes i.p.v. weefsel microarrays om te onderzoeken of de locatie van de TIL belangrijk is. Het is bekend dat de meeste immuuncellen die aangetrokken zijn door het kankerweefsel aan de rand van de tumor blijven en niet doordringen tot in de tumor. We tonen aan dat de TIL die doordringen tot in de tumor juist belangrijk zijn voor de overleving van patiënten en niet de cellen die buiten de tumor blijven. We tonen ook aan dat het belang niet zit in de absolute hoeveelheid TIL die binnendringen in de tumor maar in de proportie van het aantal cytotoxischer ($CD8^+$) immuuncellen (dat is cellen welke kankercellen bestrijden) in relatie tot het aantal regulerende ($Foxp3^+$) immuuncellen (i.e. cellen die kankercellen helpen door het immuunsysteem te remmen). Deze laatste observatie, namelijk dat kwaliteit belangrijker is dan kwantiteit van de immuuncellen in termen van overleving, komt ook terug in hoofdstuk 4 over pancreascarcinoom en papilcarcinoom, alhoewel in die studie gebruik gemaakt werd van weefsel microarrays.

In de hoofdstukken 6 en 7 maken we de overgang van kankercelexpressie van biomarkers naar mogelijke biomarkers welke aanwezig zijn in de bloedcirculatie. In hoofdstuk 6 bespreken we de bestaande literatuur over routine laboratoriumtesten, zoals aantal neutrofielen, lymfocyten en bloedplaatjes, of combinaties hiervan, als potentiele biomarkers voor overleving. In hoofdstuk 7 onderzoeken we de prognostische rol van de PD-L1 en Galectine-9 moleculen in de bloedcirculatie van patiënten met hepatocellulair carcinoom. We laten hier zien dat er een verband bestaat tussen een hogere concentratie van deze immuunremmende moleculen in de bloedcirculatie en betere kankeroverleving. Dit is vergelijkbaar met wat we zagen in hoofdstuk 3 voor de kankercelexpressie van deze moleculen. Het is echter verrassend dat de patiënten met een hoge PD-L1 en Galectine-9 concentratie in het bloed niet altijd een hoge expressie van PD-L1 en Galectine-9 in de kankercellen hebben. Dit betekent dat bloed concentratie en kankercelexpressie onafhankelijk van elkaar prognostische informatie bevatten. Patiënten met

hoge PD-L1 of Galectine-9 gehalte in zowel het bloed als in kankercellen hebben een uitstekende prognose, terwijl patiënten met lage PD-L1 of Galectine-9 gehalte in bloed en kankercellen een zeer slechte prognose hebben.

In hoofdstuk 8 onderzoeken we een aantal tumor-geassocieerde antigenen als mogelijke componenten voor toekomstige vaccins voor hepatocellulair carcinoom. We laten zien dat een panel van vier antigenen, namelijk annexine-A2, glypican-3, MAGE-C1 en MAGE-C2, tot expressie worden gebracht door kankercellen in een significant aantal patiënten, terwijl ze tegelijkertijd zeer weinig (Annexine-A2) of helemaal niet (glypican-3, MAGE-C1 en MAGE-C2) in het omringende normale leverweefsel tot expressie worden gebracht. Deze eigenschappen maken dat ze geschikt zijn als targets voor vaccinatie. Ons laboratorium is momenteel bezig met het ontwikkelen van een dergelijke vaccinatiestrategie voor hepatocellulair carcinoom. In hoofdstuk 8, in overeenstemming met de rest van dit proefschrift, blijkt eveneens dat patiënten die veel (7 of meer) van deze tumorantigenen tot expressie brengen langer overleven dan patiënten die zeer weinig (2 of minder) van deze tumor-antigenen tot expressie brengen. In de algemene discussie, hoofdstuk 9, veronderstellen we dat misschien, vergelijkbaar met de expressie van immuunremmende moleculen, de expressie van te weinig tumorantigenen kan betekenen dat de kankercellen niet meer worden gedetecteerd door immuunsysteem. En dit zou de slechte prognose van deze patiënten kunnen verklaren.

Hoofdstuk 9 is een algemene bespreking van dit proefschrift. Wij richten ons op bevindingen die een diepere discussie behoeven en verstrekken additionele hypotheses en wetenschappelijke ideeën om deze bevindingen te verklaren. We gaan daarnaast in op de technische aspecten van onze experimenten, zoals bijvoorbeeld het belang van de kwaliteit van antilichamen die worden gebruikt in immuunohistochemie en de geschiktheid van het gebruik van weefsel microarrays versus volledige weefselcoupes. Tot slot dragen we ook ideeën aan voor toekomstige vervolgstappen van dit onderzoek. Ons uiteindelijke doel is het ontwikkelen van klinisch bruikbare en immuunspecifieke biomarkers in het huidige tijdperk van kanker immunotherapie.



PART VI

APPENDIX

ACKNOWLEDGMENTS

~ No one can whistle a symphony. It takes a whole orchestra to play it ~
H.E. Luccock

Any achievement in life requires the cooperation, support and hard work of many people. It is as true for finishing one's PhD as it was true for building the pyramids. It is therefore with great pleasure that I would like to thank the people that helped me achieve this goal.

To my promotors and co-promotor

Dear **Marco**, thank you for supporting me during these years. Scientifically, logistically and materially. And for allowing me the opportunity to complete a PhD. Thank you for the time and effort that went into that, for your ideas, our discussions, your patience. In addition, I could clearly see during the clinical meetings that you do not only run a department but you are an excellent clinician, a role model, genuinely dedicated to your patients. This was important to witness and meant a lot to me.

Dear **Stefan**, thank you for all your support during the time of my PhD. It was an honor to have you as a promotor. Thank you for the thorough criticism and insights to my projects. By the way, I was always struck by the speed and dedication by which you turn around manuscripts. Despite the fact that you also run a department, it was a frequent occurrence that I would send you a manuscript only to receive it back, well read, commented and criticized, the very same day. Nothing like setting the example.

Dear **Jaap**. To say that I found you when I needed you the most is an understatement. You were the perfect supervisor for me. Not only you helped me see the light at the end of the tunnel (that came much later) but helped me find the tunnel in the first place. Your organizational skills are unparalleled. Your experience and knowledge in immunology runs deep. Your critical but most importantly practical appraisal of the literature, of the projects, of the experimental findings, is outstanding. Your dedication to your students is superb. I wouldn't be surprised if we have spent a few hundred hours between various meetings discussing projects. The fact that we often disagreed was never a limitation, as stubbornness was never an issue (at least not from your side). I would like to tell you that I have never disagreed so often and so pleasantly with anyone in my life. I always felt comfortable and confident with you by my side

To the members of my PhD committee

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Dear **Professor Ijzermans**, thank you for supporting my projects and offering constructive criticisms to both my manuscripts and to the many presentations during the LENTIS meetings. You were always encouraging. I admire your dedication in attending the many laboratory research meetings.

Dear **Professor Beuers**, thank you for supporting our collaborative projects between Erasmus and the AMC. It is my honor that you are part of my PhD committee. I appreciate the time and effort you have spent in reviewing my work.

Dear **Reno**, you are a role model for any aspiring young investigator. Your criticisms of my projects were always thorough and I looked very much forward to every presentation I ever gave in the TIP meetings. It is only now that I realize, with surprise, that despite the fact your suggestions and ideas are scattered, in little bits and pieces, throughout my thesis you are not a co-author in any of my manuscripts. This is obviously because you share your astute and critical ideas freely to all, especially in the wonderful tumor immunology platform meetings you've set up. I am sure that every participant in those meeting feels the same.

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Dear **Professor Aerts**, it is my honor that you are part of my PhD committee. Thank you taking the time to review my thesis. I would also like to thank you for your input to my work during the TIP meetings.

To colleagues instrumental for the completion of my PhD

Dear **Katharina**. What can I say. It would have been impossible to complete most of the projects in this thesis without you. I have spent countless hours in your office both discussing the scientific merits and logistics of many projects as well as looking at, surely, a few thousand slides. And this while your desk was pilling up, even as we spoke,

with more and more pathology cases from the OR to review. You dedication to scientific research is admirable. It was my pleasure and distinct honor to work with you. I would have loved for you to take part in my PhD committee but you had other plans… It is all for the best though J

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Dear **Dave**, thank you for all the constructive criticism of my work. Some say you are a difficult person to work with. … They are right ☺. Of course this is because of your dedication in critically appraising the work of your colleagues. Which is what one is supposed to do anyway. I dreaded the arrival of your comments. Every time. But every time my manuscripts significantly improved in substance. Thank you for that.

Dear **Professor Peppelenbosch**, thank you for all the advice you gave me and for supporting my projects. Your door was always open to me. Thank you for your input during the MDL meetings.

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Dear **Bettina**, thank you for spending all the time to statistically make sense of my projects. You taught me a lot and provided me with the security needed in my work. You are a pleasure to work with.

Dear **Steven**, thank you for the dedication you showed during your master thesis in our HCC project. Constructing those first TMAs and setting up all those immunohistochemi-

cal assays felt like pioneering work (to us at least ☺). It was a rewarding experience and your help, especially in those early and challenging days, was invaluable. I miss working with you very much.

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To colleagues from the Academic Medical Center

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Dear **Bart**, thank you for helping me complete our HCC database and for all the phone calls you made to collect information. Thank you for your enthusiasm and support of my projects in general.

Dear **Gerrit**, thank you for helping me with the TMA construction at the AMC. Your input was invaluable. You were a pleasure to work with.

To colleagues from the MDL lab

Dear **Estella**, thank you for all your contributions and input to my projects. I felt we were two parallel lines moving side by side and at some point we (our projects) were supposed to meet (just like in Lobachevskian geometry – google it). Somehow, that never happened. I blame Jaap (he won't read that far in the acknowledgments, don't worry)! In any case it was wonderful working with you, you are very smart, and wish you the very best.

Dear **Abdullah**, thank you for all your patience sitting through the numerous conferences and presentations of our group. You were always engaging and your comments were always insightful. It was a pleasure to work and publish with you.

Dear **Sonja**, thank you for helping me understand (or so I think) new analytical approaches and possibilities in proteomics. You certainly opened my eyes to new horizons. In addition, your expertise on Galectin-9 could not have come at a better time. Thanks for all the discussions.

Dear **Angela**. I will acknowledge you here, right after your friend above, since there is no special "Nijmegen" section. Thank you for tirelessly helping Xiaolei and me with inForm and Vectra. It was fulfilling to witness the possibilities (and complexities) of new imaging technologies. Even in your very last day at work, and with so much to do, you were helping us until late in the day. I am happy we can continue our collaboration while at your new job in Paris. Wish you all the best.

Dear **Kim**, thank you for teaching me immunohistochemistry. You had the uncanny ability to walk into the lab at precisely the wrong moment. I had to look over my shoulder for a couple of years in the lab to see if you're coming, as well as double and triple check that the microscope light was shut off (even in days I wasn't using it!). Thanks for helping me out.

Dear **Hannah**, you showed exemplary dedication during the TMA scorings and I thank you very much for that. We've spend many ours on the microscope even when you were busy with your clinical rotations. I appreciate your help very much.

Dear **Ron**, thank you for your participation and dedication to our many group meetings and presentations. I could always count on you for insights. You have a way to make criticism sound always sweet. Your own presentations are always enjoyable and educational.

Dear **Luc**, thank you for your active participation during the LENTIS meetings. You critically appraised my work and contributed to many of our ideas.

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To colleagues from the Pathology department

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Dear **Paulina**, thank you for retrieving thousands of slides and blocks for my projects.

To the TIP meeting participants

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To the secretarial staff

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To colleagues from the Mayo Clinic

Dear **Haidong** and **Susan**, thank you very much for your help with the circulating ligand project. Your input has been invaluable to us. I hope very much we can continue our collaboration in the future.

Xavi, my friend. Thank you for your help with the 5H1 antibody and getting me into contact with Haidong and Susan. I have read your papers many times and refer to them quite a bit.

I would also like to thank my old colleagues from the department of oncology at Mayo Clinic who trained me, inspired me and made me the doctor I am today. Some special mentions:

Dear **Charles**, thank you for believing in me, recruiting me from residency and mentoring me during those wonderful years. I owe a lot to you.

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Dear **Keith**, thank you for being my mentor and role model. I still can't be at the lab at 5 in the morning though…

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To my paranymphs

Mike, my friend. It is my honor that you come all the way from the United States to be present at my PhD defense (the other guy is only coming from the border with Belgium by the way). We've been through a lot together and it means the world to me that you are here. Perhaps I can repay you the honor one day. By coming to your wedding perhaps? Dear **Ana**, thanks for letting this guy come over. It means a lot to me. But you already know that. I love you guys very much.

Dear **Alexander**, thank you for being by my side during such important a moment. It is my pleasure and great honor. **Monika**, yourself and the kids are like family to us. I hope to continue our friendship for many years to come.

To friends in the Netherlands

Dear **Tirza**, **Tjard**, **Marije** and **Yuk Keung**, thank you for being there for us in the Netherlands. Your friendship means the world to us.

To my parents

Dear **mama** and **baba**. Your input into my PhD thesis was done a very long time ago. It was when almost everybody else on this long list of people I am thanking today was either a child or not born yet. It was when you set the foundations of my being, my principals, my character. And when you paid a lot of money for my education, buying dollars with olive oil (both of these things meant something back in those days). Thank you for everything you've ever done for me. Although, you would have done it anyway. It is my immense pleasure that you are present at my defense.

Dear **mama** and **baba**. People usually gain new children with time, not new parents. I got both. The support, richness and love you provide to us and the kids every day is beyond comprehension. I am happy to live by your side and I am very proud of you both. I only hope we can return back to you a fraction of what you have sacrificed during your lives. But that is impossible. And one request: If I could only see you to complain once, … just once.

To my brothers and sisters

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To my grandmother

Αγαπητή **γιαγιά**. Από τα παιδικά μας χρόνια γέμιζες τη ψυχή μας με αγάπη και φώς. Τώρα μπορούμε και δίνουμε λίγο από αυτό το φώς στα δικά μας παιδιά. Σε σκέφτομαι και σε αγαπώ πάρα πολύ.

To my children

You are the light I see and the air I breathe.
Erin ... λέγονται στρουμφάκια, οχι στραβούκια.
Alex, ὰμα σι πιάσου...

To Sarwa

On we go with that trip we started 13 years ago. We both, at different times, sacrificed our present for a future together. We both struggled to adjust, to adapt. Are we there yet? Do you regret anything? It's been like a dream. But I don't want to wake up. Not now. Not ever. So, forward we go to the next chapter. What will this one bring? I don't know. All I know is that as long as you're standing by my side I have nothing to fear and everything to look forward to.

PHD PORTFOLIO

Name of PhD student	Kostandinos Sideras
Erasmus MC departement	Gastroenterology and Hepatology
PhD period	March 2012 – December 2016
Promotor	Prof. dr. M.J.Bruno
Promotor	Prof. dr. S. Sleijfer
Co-promotor	J. Kwekkeboom

General courses

Basic Introduction Course on SPSS	2012
Biostatistical Methods I: Basic Principles (CC02)	2013
Introduction in GraphPad Prism	2015
Program Basic Human Genetics course: Genetics for Dummies	2015
Workshop on InDesign CS5	2015
Photoshop and Illustrator CS6 Workshop	2015

Conference and symposium attendance

American Society of Clinical Oncology (ASCO), Chicago	2012
Association for Cancer Immunotherapy (CIMT), Mainz	2013
Exploring New Methods and Imaging Technologies for Cancer Immunotherapy Research Pathology Imaging User Group (Perkin Elmer), Rotterdam	2014
Nederlandse Vereniging voor Gastroenterologie (NVGE), Veldhoven	2016
Minisymposium: 'HCC: nieuwe therapieën', Rotterdam	2016

Seminar and meeting attendance

Tumor Immunology Platform (TIP) meeting	2012-2016
MDL Seminar	2012-2016
Gastrointestinal Oncology research meeting	2015-2016
LETIS/MDL research meeting	2012-2015

Conference presentations

The intra-tumoral ratio of regulatory T cells to cytotoxic cells predicts recurrence following hepatic metastatectomy of patients with colorectal cancer - Association for Cancer Immunotherapy (CIMT) – poster presentation	2013
Tumor antigen expression differs in hepatocellular carcinoma (HCC) between viral hepatitis and non-viral hepatitis patients. - Digestive Disease Week (DDW) – poster presentation	2014
Tumor antigen expression in HCC in a low-endemic western area Nederlandse Vereniging voor Gastroenterologie (NVGE) – poster presentation	2015
Under-expression of PDL1, Galectin-9 and CD8 TIL in Hepatocellular Carcinoma is associated with worse patient survival - Nederlandse Vereniging voor Gastroenterologie (NVGE) – oral presentation	2016
Tumor expression of immune inhibitory molecules and TIL counts predict pancreatic cancer survival - Association for Cancer Immunotherapy (CIMT) – poster presentation	2016

Selected Lectures

Hepatocellulair carcinoom 2012: State of the Art and future strategies. Guidelines and screening of liver tumors (invited), Rotterdam	2012
Liver metastasis in Colorectal cancer – LETIS/MDL meeting	2013
Tumor infiltrating lymphocytes in CRC metastasis – MDL seminar	2013
CIMT conference update – TIP meeting	2013
ASCO 2013 Tumor Immunology Update – TIP meeting	2013
HCC antigen expression – Gastrointestinal Oncology research meeting	2014
SMAD 4 - Gastrointestinal Oncology research meeting	2014
HLA-G expression in HCC- Gastrointestinal Oncology research meeting	2014
Galectin-9 expression in HCC - Gastrointestinal Oncology research meeting	2014
IDO expression in HCC - Gastrointestinal Oncology research meeting	2015
Galectin 9 in Cancer - TIP meeting	2015
Papers and Grants – MDL seminar	2015
Immune Inhibitory ligand and enzyme expression in HCC - TIP meeting	2015
Immune inhibitory ligand expression in pancreas cancer - Liver Cancer Meeting	2015
Immunologic escape mechanisms in liver and pancreatic cancer - MDL seminar	2015
Cytotoxic and targeted therapies in GI malignancies (invited), Rotterdam	2016
Circulating PD-L1 and Galectin-9 - TIP meeting	2016

Teaching

Meet the Expert; Translationeel onderzoek HCC: Medical student lecture	2013
Supervising Master Thesis: Steven J. Bots	2014
Supervising Master Thesis: Kevin Yap	2015
Research Master Infection & Immunity: student lab rotation lecture	2015

Peer reviewed submitted papers for various scientific journals including Oncoimmunology, Transplant International and European Surgical Research.

ABOUT THE AUTHOR

Kostandinos Sideras was born on July 11th 1974 in Brooklyn, New York. At the age of 6 he moved with his family to the island of Lesbos in Greece. There he completed his primary education. In 1993 he moved back to the United States. He attended the State University of New York at Stony Brook, where in 1998 he attained his bachelors diploma, with Summa Cum Laude and departmental honors, in Chemistry. During this time he also completed a minor in History, was awarded the Sei Sujishi Prize for Outstanding in Freshman Chemistry as well as the Outstanding in Senior Chemistry award. In 2002 he attained his Medical Diploma with Recognition in Research from the same university. Thereafter he moved to the Mayo Clinic in Rochester Minnesota where in 2005 he completed his residency in Internal Medicine and in 2009 Fellowships in Hematology and Oncology. During his senior year he was awarder with the Outstanding Trainee in Oncology award. He joined staff at the Department of Oncology at the Mayo Clinic in 2009 with a focus on Breast Cancer, Thyroid Cancer and cancers of unknown primary site. He was a member of the North Central Cancer Treatment Group (NCCTG) scientific committee as well as a member of the Translational Breast Cancer Research Consortium (TBCRC). He has initiated and published several treatment clinical trials and he is also the author of the book chapter on Oral Complications in the 4th and 5th editions of Abeloff's Clinical Oncology. In 2012 he moved with his spouse, currently a transplant hepatologist, to Rotterdam where his daughter and son were born. There, at Erasmus University Medical Center, and under the supervision of prof. dr. M.J. Bruno (Department of Gastroenterology and Hepatology) and prof. dr. S. Sleijfer (Department of Medical Oncology), as well as his co-promotor Jaap Kwekkeboom (Department of Gastroenterology and Hepatology), he completed this PhD thesis.