Towards a tailored therapeutic approach for vulvar cancer patients

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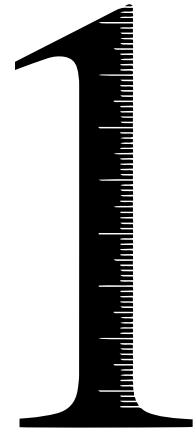

INTRODUCTION

Precision medicine refers to tailoring of medical treatment to the individual characteristics of each patient. For over 60 years, health care providers used a staging system that includes the size of a tumor, involvement of lymph nodes, and distant metastasis. Based on the stage of disease, patients were offered information on the therapeutic options and prognosis. But the clinical outcome of patients varies within the same stage of disease, which illustrates that the staging systems may not provide optimal prognostic information. Integration of cellular and molecular variables in staging systems has led to refinement in prognosis of various tumors.[1-3] Current cancer care is slowly transitioning from a 'one size fits all' approach towards precision medicine. Consequently, refinement of individual prognostic estimates occurs, and tailored (new) therapeutic strategies are proposed to improve mortality rates and diminish unintended treatment-related morbidity.

Vulvar cancer is a rare gynecological malignancy and falls by the wayside when it comes to implementing the concept of precision medicine. Mainly elderly patients are affected by this. Especially because elderly patients are affected by this disease[4], therefore treatment decisions must be individualized with regard to co-morbidity, low complication rates, and optimal treatment.[5, 6]

The aims of this thesis were to gain insight in the molecular alterations of vulvar cancer, to identify prognostic markers to refine clinicopathological risk assessment, and to undertake a first step to broaden the spectrum of treatment strategies with immunotherapy. This chapter summarizes the clinical background and current knowledge on the molecular and cellular aspects of vulvar cancer that may impact prognosis.

VULVAR SQUAMOUS CELL CARCINOMA

Vulvar squamous cell carcinoma (VSCC) is the most common histological type of vulvar cancer.[7] According to the Dutch Cancer Registration, 362 women were newly diagnosed with VSCC in 2017 in the Netherlands. This is an imposing increase compared to 1990, in which 168 women were diagnosed with VSCC (figure 1). Although VSCC mainly affects elderly women of 60-70 years[4, 5, 7], incidence rates are also increasing in younger women (figure 1, dashed line).[4]

VSCC are often initially misdiagnosed by doctors as inflammatory conditions, delaying diagnosis and worsening prognosis. VSCC can present itself as a raised, flat ulcerated plaque-like, or warty mass on the vulva. Although patients can be asymptomatic at diagnosis, most patients experience pruritus, burning, pain, discharge, or bleeding.[8]

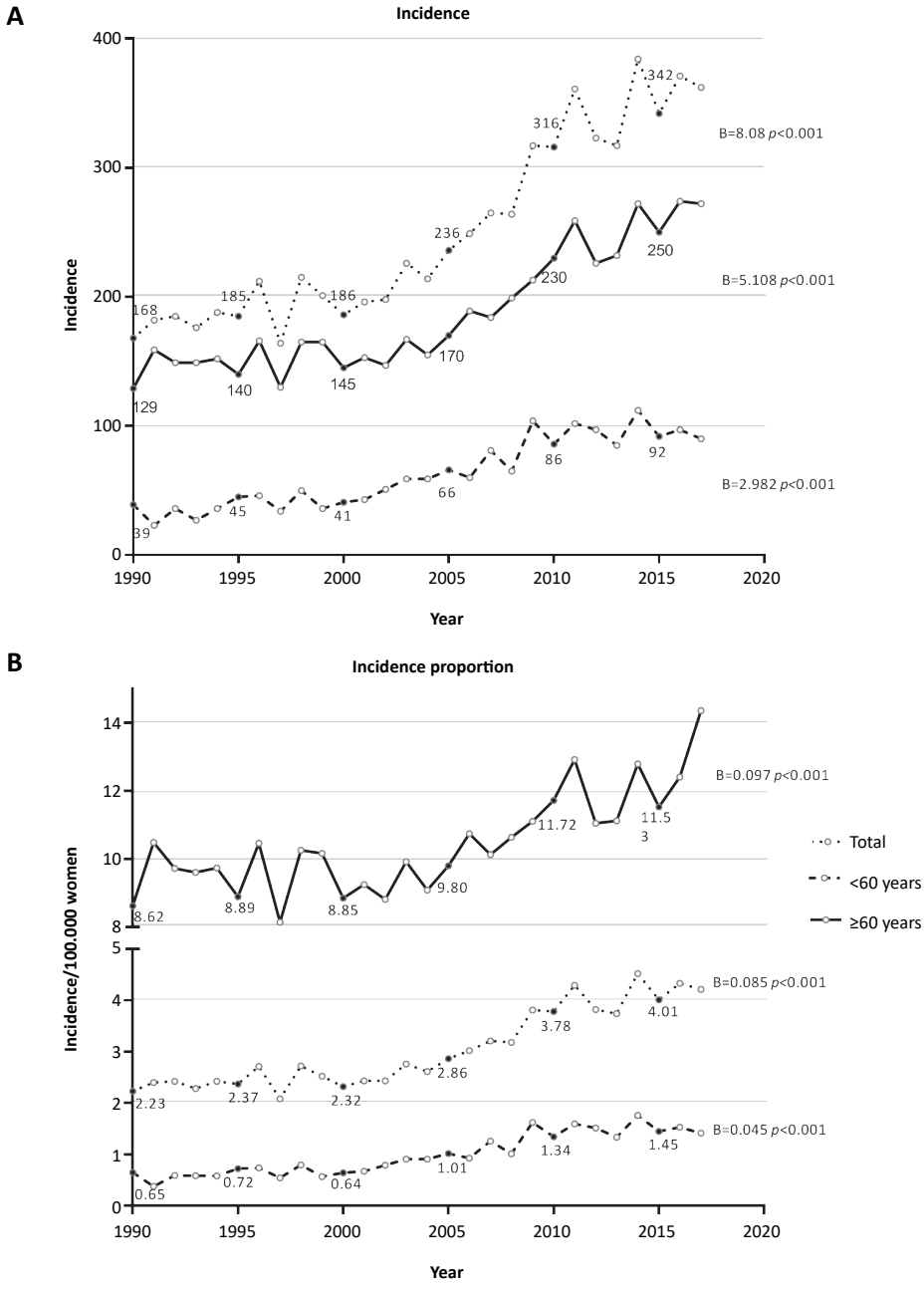

Figure 1. The incidence and incidence proportion of vulvar squamous cell carcinoma from 1990-2017 in all patients, and stratified for age with a cut-off at 60 years of age. The total number of patients are shown in the dotted line. Patients below 60 years of age represent the dashed line, and older than 60 years of age are depicted in the solid line. Both age categories showed an increased number of cases for both the incidence (panel A) and incidence proportion per 100.000 women (panel B). Data retrieved from the Dutch Cancer Registry.

Different molecular subtypes of VSCC

VSCC can develop via at least two separate etiological pathways. One is associated with a persistent infection of high-risk human papillomavirus (hrHPV) and accounts for approximately 20% of all VSCC, but the majority of VSCC is HPV-independent.[9-11]

HPV-associated vulvar squamous cell carcinoma (HPVpos VSCC)

HPV is a small DNA virus that infects the basal cells of squamous epithelium when the epithelial surface is disrupted through minor damage of the genital mucosa. This allows access for the virus to enter the basal cells of the epithelium.[12] Over 100 types of HPV are identified which are subdivided into low-risk HPV (non-oncogenic) and high-risk HPV (oncogenic).[13] The lifetime risk of acquiring a hrHPV infection is approximately 80% for sexually active individuals. The great majority of the infections are cleared by the immune system within 18 months.[14] Less than 10% of the infections persist and may cause a precursor lesion of HPVpos VSCC, called vulvar high grade squamous intra-epithelial lesion (vHSIL), formerly referred to as vulvar intraepithelial neoplasia of the usual type (uVIN).[15] Even after treatment, 3-4% of the vHSIL patients will subsequently develop VSCC.[15, 16] Almost 75% of HPVpos VSCC lesions are caused by HPV type 16, one of the most dominantly present oncogenic hrHPV in the Western world.[17]

HPV-independent vulvar squamous cell carcinoma (HPVneg VSCC)

The non-virally induced VSCC (HPVneg VSCC) are most common (80%)[9-11], and arise from a precursor lesion called vulvar intraepithelial neoplasia of the differentiated type (dVIN).[18] Lichen sclerosus (LS) is often noticed in the background of HPVneg VSCC, although the precancerous potential of lichen sclerosus is uncertain.[11, 15] HPVneg VSCC is thought to be associated with *TP53* mutations (HPVneg/p53mut VSCC), which leads to abnormal expression of the p53 protein.[11, 19, 20] Somatic mutations in *TP53* lead to an uncontrolled cell cycle and chromosomal instability, resulting in tumor formation.[21] Recently, a third distinct molecular subtype was proposed which contains HPVneg VSCC without *TP53* mutations (HPVneg/p53wt VSCC).[22] Ten HPVneg/p53wt VSCC were analyzed with targeted next-generation sequencing and showed mainly mutations in *NOTCH1*, *HRAS* and to a lesser extent *PIK3CA*.[19] It is possible that these mutations are the drivers for the oncogenesis in HPVneg/p53wt VSCC[19], as precursor lesions of HPVneg/p53wt VSCC such as differentiated exophytic vulvar intraepithelial lesion (DeVIL) and vulvar acanthosis with altered differentiation (VAAD) are both bearing mutations in these genes.[19, 23, 24]

FIGO staging of VSCC

VSCC is staged using the International Federation of Gynecology and Obstetrics (FIGO) staging system. This classification system is based on clinical and histological parameters such as the size of the tumor, depth of invasion, nodal metastasis, and distant metastasis (figure 2). The

criteria for adjuvant treatment are dependent on histopathologic evaluation after resection of the tumor by measuring stromal invasion and assessing the number of node metastases, extra-capsular spread, and whether the tumor invades structures as the upper urethra, vaginal mucosa, bladder mucosa, rectal mucosa, pelvic bone, and/or inguinofemoral nodes.[25]

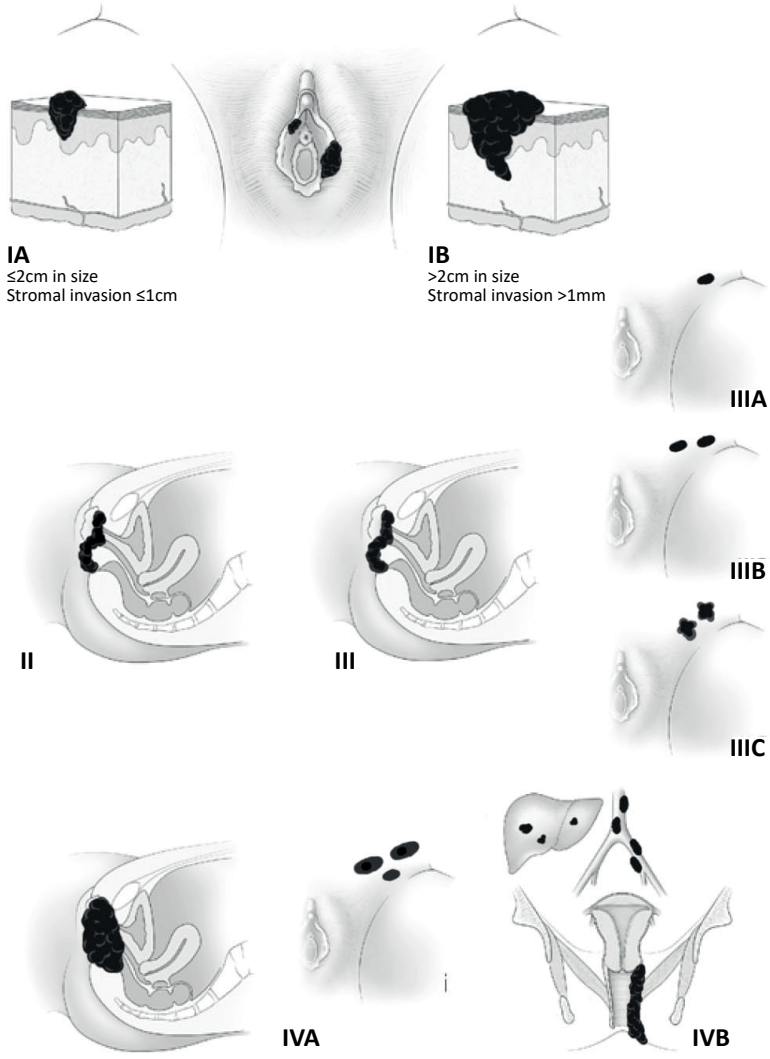

Figure 2. Staging vulvar squamous cell carcinoma (FIGO 2009). Adapted from Williams Gynecology, 3rd Edition. The International Federation of Gynecology and Obstetrics (FIGO) staging system for vulvar cancer.

Treatment and prognosis

The standard treatment for VSCC is a wide local excision (WLE) with excision margins of at least 1-2cm. When the depth of invasion is ≤1mm, patients are treated with a WLE only, because groin metastasis almost never occur in this stage.[26-28] However, in case of a depth of invasion of >1mm, a lymphadenectomy or sentinel lymph node (SLN) procedure is recommended. In case of a unifocal tumor of ≤4cm, a SLN is recommended instead of lymphadenectomy. When a tumor is >4cm or multifocal, a WLE and inguinal lymphadenectomy will be performed. Adjuvant radiotherapy may be indicated in case of positive tumor margins and/or lymph node metastasis to improve prognosis.[29, 30]

The prognosis of early-stage (FIGO I/II) VSCC without lymph node metastasis is excellent (80-90% 5-years survival). But the 5-year survival decreases to 24-75% depending on the size and number of lymph node metastases.[31] VSCC are notorious for high recurrence rates, with a reported local recurrence frequency up to 40% ten years after primary treatment.[32] Groin recurrences occur in 9-38% and distant metastasis in 8% of the patients.[33] Prognostic factors for recurrent disease such as tumor size, stromal invasion[34], and a histological resection margin of ≤8mm have been described.[35] The latter is still debated[36], although some guidelines adhere strictly to this cut-off and recommend re-excision in case of a surgical margin of <8mm to lower the risk on recurrent disease.[37] In some cases, re-excision is not an option and postoperative radiation is recommended to reduce the local recurrence rate and improve overall survival.[38] It is vital to assess the cut-off of this surgical margin to promote minimal tissue removal with consequently lower morbidity and complication rates[39] without compromising clinical outcome.

The prognosis of VSCC has not improved over the past decades (table 1) and postoperative morbidity remains a lingering burden.[7, 26, 27, 35, 40-43] To boost the development of innovative and less mutilating forms of treatment, we studied the potency of precision immunotherapeutic treatment strategies for VSCC. First, we summarized the interplay between cancer and the immune cells by the cancer-immunity cycle, followed by a description of the mode of action of various immunotherapeutic agents.

Table 1. Overall- and relative survival percentages per age group of vulvar squamous cell carcinoma throughout decades.

Age	1990-2017 (*n*=7007)	1990-1999 (*n*=1876)	2000-2009 (*n*=2351)	2010-2017 (*n*=2780)
5-year overall survival				
20-39	85.5% (80.4-89.4)	82.1 (71.2-89.2)	81.0% (71.5-87.6)	94.9% (87.0-98.1)
40-49	87.7% (84.6-90.2)	83.8% (75.8-89.3)	88.6% (83.5-92.2)	88.5% (82.9-92.3)
50-59	79.3% (76.2-81.9)	78.9% (71.8-84.4)	78.6% (73.4-82.9)	80.3% (75.6-84.2)
60-69	71.4% (68.8-73.9)	72.6% (67.6-76.9)	68.7% (64.0-73.0)	72.0% (67.5-76.0)
70-79	58.8% (56.5-61.0)	57.4% (53.4-61.3)	58.5% (54.6-62.2)	60.3% (56.1-64.2)
≥80	29.0% (27.0-31.1	29.4% (25.7-33.2)	26.4% (23.3-29.7)	31.1% (27.4-34.9)
<60	83.2% (81.2-84.9)	81.2% (76.8-85.0)	82.5% (85.3-79.2)	84.8% (81.7-87.5)
≥60	50.3% (48.9-51.6)	50.5% (48.0-53.0)	47.9% (45.6-50.2)	52.0% (49.5-54.4)
5-year relative survival				
20-39	85.8% (80.7-89.7)	82.5% (71.5-89.6)	81.3% (71.8-87.9)	95.2% (87.2-98.3)
40-49	88.5% (85.4-91.1)	84.7% (76.6-90.3)	89.5% (84.3-93.1)	89.2% (83.6-93.0)
50-59	81.0% (78.0-83.8)	80.9% (73.6-86.6)	80.4% (75.1-84.8)	81.9% (77.1-85.9)
60-69	75.5% (72.8-78.2)	77.6% (72.3-82.3)	72.5% (67.5-77.1)	75.7% (70.9-79.9)
70-79	69.4% (66.7-72.0)	69.7% (64.8-74.4)	69.0% (64.4-73.4)	69.2% (64.4-73.7)
≥80	55.3% (51.4-59.3)	59.2% (51.7-66.8)	50.3% (44.2-56.6)	57.2% (50.4-64.2)
<60	84.4% (82.4-86.2)	82.5% (78.0-86.3)	83.3% (80.4-86.6)	86.0% (82.8-88.7)
≥60	66.1% (64.3-67.9)	68.2% (64.8-71.5)	63.2% (60.1-66.3)	66.8% (63.6-69.9)

Percentages followed by 95% confidence interval. OS = overall survival, RS = relative survival. Data were retrieved from the Dutch Cancer Registry.

CANCER-IMMUNITY CYCLE

The immune system and the development of cancer are intertwined. This is supported by the observation that people with immune deficiencies often develop tumors, in particular virally-induced tumors.[44-46] The tumor microenvironment (TME) may be infiltrated with several types of immune cells, the composition of those cells may have impact on clinical outcome after standard treatment.[47] The most frequently described cells which mediate antitumor reactivity are $CD4^+$ T helper 1 (Th1) cells, $CD8^+$ T cells, natural killer (NK) cells, dendritic cells (DCs), type 1 macrophages, and inflammatory myeloid cells. In contrast, the presence of myeloid-derived suppressor cells (MDSCs), type 2 macrophages and regulatory T cells (Tregs) dampen antitumor responses and are associated with worse clinical outcome.[48] The steps that are needed for an effective anti-cancer response are summarized in the cancer-immunity cycle.[49] Suboptimal performance in either one of the seven indicated steps, leads to the inability of the immune system to optimally recognize, kill, and control cancer cells.[49] Therefore, cancer cells use different strategies to interfere with every step of the cycle for instance by loss of antigenicity, loss of immunogenicity, and/or creating an immunosuppressive TME.[50]

The first step in this cycle is the release of antigens (step 1, figure 3), which can be categorized as tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs). TSAs are exclusively expressed by cancer cells and are therefore considered to be more potent as therapeutic

targets since they are foreign to the body.[49] One example of TSAs are the oncogenic proteins from HPV. Another example is the formation of neoantigens as result of somatic mutations in cancer cells, which are unique to an individual patient. TAAs are non-mutant molecules that are aberrantly expressed in cancer cells, but often also found in healthy tissue. They may be less effective antigens since T cells might either not recognize these TAAs with high affinity because of central tolerance, or the responding T cells may cause severe autoimmunity.[51]

The next step in the cycle is to process these cancer antigens and subsequently present them in the context of a major histocompatibility compex (MHC) molecule at the cell surface of antigen presenting cells (APCs, steps 2 &3). The presentation of the antigen from APC to naïve T cell requires three signals: 1) interaction of the MHC-antigen complex with the T cell receptor (TCR)[52], 2) co-stimulation for instance by interaction between co-stimulatory receptors CD28 and CD27 with ligands CD80/CD86 and CD70[52, 53], respectively, and 3) the secretion of cytokines (e.g. IL-12 and IFN-α) by the APC.[52] To combat cancer cells, both $CD8^+$ T cells and $CD4^+$ Th1 cells must migrate towards the tumor (step 4), and infiltrate the tumor (step 5) in order to exert their anti-tumor function.[52] It is known that $CD4^+$ Th1 cells are needed to promote and sustain a $CD8^+$-mediated T cell response, which is most effective in killing tumor cells.[54] After all previous steps have successfully occurred, antigen recognition after cross-linking with the TCR (step 6), leads to lysis of cancer cells by $CD8^+$ T cells' secretory granules (step 7).[52] Apoptosis of cancer cells releases supplementary tumor antigens, which provides a positive feedback loop to promote an anti-tumor immunity (step 1).[49]

All consecutive steps of the cancer immune cycle need to be fulfilled to combat cancer cells. However, cancer cells may interrupt the cycle at one or more of these steps and thereby allow the cancer cells to escape from immune control. It is known that some cancer cells may have lower expression of antigens, and will therefore evade recognition by the immune system. Consequently, natural selection occurs and progress into less antigenic tumors.[50] In case cancer cells do secrete antigens, APCs (and specifically DCs) should be recruited and activated by for instance danger-associated molecular patterns (DAMPs). In tumors, however, myeloid cells can differentiate towards MDSCs and tumor-associated macrophages (TAMs) due to cytokines and chemokines produced by cancer cells. This may hamper an anti-tumor response by suppressing the activity of T cells, NK cells, and DCs.[55] Moreover, the antigen presentation of the tumor cells may be hampered by defects in the antigen processing machinery or due to downregulation of MHC expression.[50] The last mentioned is often described in HPV-associated cancers.[56] Another hurdle to overcome an adequate anti-tumor response, is the upregulation of programmed death ligand-1 (PD-L1) molecule on the cell surface of cancer cells after stimulation with interferon γ (IFN-γ) that is produced by tumor infiltrating lymphocytes (TILs) as this may inhibit the function of effector T cells.[57] In addition, other molecules including cytokines and chemokines secreted by cancer cells may sculpture a suppressive microenvironment by recruiting MDSCs, TAMs, tumor-associated

neutrophils (TANs), and Tregs.[52, 58] In addition, T cells express cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) which binds to B7 molecules on APCs, upon binding T cell tolerance is induced. Another example of an immunomodulatory enzyme that leads to T cell suppression is indoleamine 2,3-dioxygenase (IDO), which is produced by some tumors in response to an interpheron gamma (IFN-γ) oriented type 1 immune response.[59]

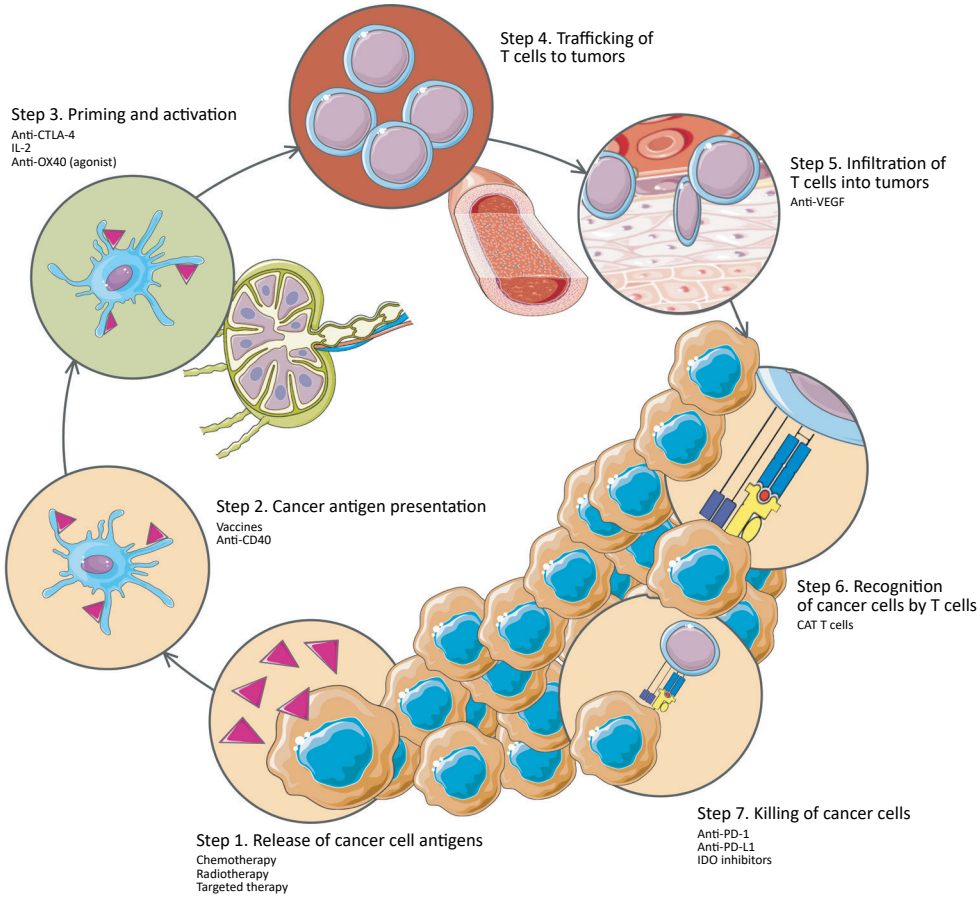

Figure 3. Schematic overview of the cancer immunity cycle (step 1-7) accompanied by immunotherapeutic strategies per cell cycle step. Illustration created from adapted images of Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Unported License.

IMMUNOTHERAPEUTIC STRATEGIES IN CANCER

The recognition that the immune system plays a dual role in cancer by suppressing or promoting tumor progression dates back to 1891. William B. Coley, who was a surgeon in New York, injected streptococcal bacteria into inoperable tumors of cancer patients. He described that the infection resulted in shrinkage of the malignant tumor, and was one of the first to demonstrate the potential of using the immune system to combat cancer.[60] This is still the main aim of cancer immunotherapy; generating a durable anti-cancer immune response by conquering the negative feedback mechanisms that inhibit the cancer-immunity cycle. Throughout the years, different immunotherapeutic strategies emerged that direct the immune system to attack cancer cells, including vaccines, adoptive T cell therapy (ACT), and checkpoint inhibition, in order to target different steps in the cancer-immune cycle with the aim to enhance an anti-tumor immune response.[61, 62] At the moment, different forms of immunotherapy have improved from a promising therapeutic strategy towards a robust clinical reality in a variety of (advanced) tumors.[63]

Non-specific immunotherapy (cytokines)

The two most common non-specific immunotherapies are interferons and interleukins. Both help to produce cells to destroy cancer cells with pleiotropic effects. Interferon-alpha (IFN-α) and interleukin-2 (IL-2) are most commonly used in cancer treatment. Soluble IL-2 was the first agent used to reverse the anergy of T cells.[64] IL-2 can be used as monotherapy (complete response rate 7% in metastatic renal carcinoma)[64], but the combination with other anticancer immunotherapy such as ACT (50% clinical response rate in metastatic melanoma), and antigen-specific vaccination (16% clinical response rate in advanced melanoma)[65] has been described to be promising in metastatic melanoma and renal carcinoma.[66] Most common adverse events with cytokine therapy are fever, fatigue, diarrhea, nausea and anorexia. Up to 10% of the patients will develop thrombocytopenia, leukopenia, hyperthyroidism or hypothyroidism. Serious adverse events are pleural edema, hypotension, autoimmune disease, neurotoxicity, and myocarditis.[67]

Another non-specific immunotherapeutic agent which is commonly known in the field of gynecology is imiquimod, a topical creme which unleashes an IFN-γ oriented innate immune response.[68] A retrospective study with vHSIL reported on 20-81% complete responsiveness, and 10-75% partial responses without serious side effects.[69] A randomized controlled trial (RCT) showed an objective clinical response in 81% of the vHSIL patients compared to the placebo group.[70]

Monoclonal antibodies

Monoclonal antibodies (MoAbs) are produced *ex vivo* and have different modes of action. The first generation of MoAbs could bind to molecules expressed on or released by tumors.

Examples are the antagonist of vascular endothelial growth factor (VEGF), which thereby prevented neo-angiogenesis and is approved for many tumor types.[71] Other types of MoAbs bind to cell surface molecules. The main aim is to deplete tumor cells via antibody dependent cytotoxicity mechanisms, such as rituximab, an antagonist of CD20 expressed by B cells to treat non-hodgkin disease.[72] Another option is to sensitize them for drugs by interfering with tumor signaling pathways. For instance, Herceptin®, a humanized Mab against Her2/Neu receptors, to treat Her2 overexpressing breast cancer.[73] These MoAbs are still used in clinics, most frequently in combination with other therapeutic agents.

More recently discovered MoAbs stimulate an adaptive immune response, because they block the interaction between molecules involved in the normal inhibition of immune responses, a mechanism that is often hijacked by tumor cells.[74] The first clinically targeted receptor blocked a T cell inhibitory molecule (checkpoint molecule), called cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). The use of anti-CTLA-4 therapy attenuates T cell activation. Another important checkpoint molecule is programmed death-ligand 1 (PD-L1), which is commonly overexpressed by cancer cells and immune suppressive myeloid cells in the tumor microenvironment and binds to programmed cell death protein 1 (PD-1) when expressed on activated TILs. By preventing the interaction of PD-1 with PD-L1 using anti-PD-1/PD-L1 immunotherapy (e.g. nivolumab, pembrolizumab), the function of the TILs are retained.[75, 76] PD-1/PD-L1 inhibitors are approved by the food and drug administration (FDA) for treating melanoma, non-small cell lung cancer (NSCLC) and other malignancies.[77] It is known that PD-1 is highly upregulated in VSCC, however clear distinction between HPVpos VSCC and HPVneg VSCC have not been made.[78, 79] Three studies reported on the effectiveness of anti-PD-1 therapy in VSCC patients[80-82] with a response rate of 20% (*n*=5)[80] and a progression free survival benefit of 3.1 months and overall survival benefit of 3.8 months (*n*=18).[81] The main reported treatment-related adverse events were fatigue, nausea, pruritis, nausea, rash, arthralgia, decreased appetite, diarrhea, and constipation.[81] These studies concluded that pembrolizumab (*n*=19) and nivolumab (*n*=5) are safe and effective to use in VSCC treatment.

Despite the impressive clinical success of immune checkpoint inhibition, tumor intrinsic- and extrinsic (immune suppressive cells) resistance remains a challenge. Therefore, blocking of other inhibitory immune receptors may complement first-generation immunotherapies to prevent or overcome resistance.[83] For instance, T cell immunoglobulin- and mucin-domain containing molecule 3 (TIM-3) and lymphocyte activation gene 3 (LAG-3) are targets of interest. TIM-3 is expressed by multiple cell types such as T cells, myeloid cells, and NK cells and is associated with regulation of immune responses.[84] By blocking the TIM-3 pathway, T cell exhaustion may be prevented and cancer immunity is enhanced together with increased production of IFN-γ in T cells. TIM-3 inhibitors are now tested as monotherapy and polytherapy in advanced (relapsed) solid tumors.[85] LAG-3 mainly suppresses T cell activation

and cytokine secretion, and has been shown to be effective as mono- or polytherapy in renal cell carcinoma and metastatic breast carcinoma. Both TIM-3 and LAG-3 targeted immunotherapies show synergistic effects in patients with PD-1 resistance.[85, 86]

Another novel immune checkpoint is NKG2A, which can be expressed on both NK cells and activated $CD8^+$ T cells. Blockade of this molecule has been shown to improve PD-1 therapy as well as tumor vaccination and ACT in mouse models.[87, 88] In head and neck cancer, an objective response rate of 31% is described after treatment with a combination of monalizumab (anti-NKG2A) and cetuximab (anti-PD-1).[88]

Oncolytic virus therapy

Oncolytic viruses are tumor-selective and will not harm healthy cells. By replicating in cancer cells, they induce apoptosis but they also activate the immune system. The release of TAAs and TSAs by apoptosis, the release of danger signals, the destruction of vascular supply, and the recruitment of inflammatory cells will benefit the induction and boosting of antitumor immunity.[89, 90] One oncolytic virus, named talimogene laherparepvec (T-VEC), has been FDA-approved for the treatment of melanoma with clinical success (complete response rate 61.5%) in early metastatic melanoma. In this study, all patients had adverse events such as influenza like symptoms, fatigue, decreased appetite, nausea, chills, pain in extremities, discomfort of the injection site, and myalgia.[91]

T cell therapy

ACT starts with the collection of immune cells from the patient's blood or tumor tissue. Consequently, the immune cells are selected for tumor specificity, and expanded, after which the activated T cell are re-infused to the patient. In late-stage metastatic melanoma an average objective response of 50% was reached and a complete response in 13% of the patients.[92, 93]

To increase the percentage of infused cells that can recognize the tumor cells, also other approaches for ACT were developed.[94] T cells derived from peripheral blood mononuclear (PBMC) were genetically modified by viral transduction of TCRs capable of recognizing tumor antigens.[95] A third approach consisted of genetically modified T cells that express an artificial chimeric antigen receptor (CAR T cell) that provided these T cells with a receptor that could bind an antigen and activate the T cell.[75, 96]

Cancer vaccines

Vaccination provides antigenic stimulation in order to boost a tumor-specific T cell response against TAAs or TSAs. Especially therapeutic vaccination strategies with different delivery systems such as recombinant viral vector-, peptide-, or protein-, nucleic acid-, and cell-based therapeutic vaccines targeting the HPV16 E6 and/or E7 antigens have been explored

clinically.[97] HPV16 synthetic long peptide (HPV16 SLP) vaccines have shown partial and complete regression of vHSIL lesions.[98-100] The use of a topical immunomodulator called imiquimod followed by HPV vaccination, led to clearance of the vHSIL lesion in 60% of these patients.[101] A recent study combined standard of care chemotherapy with HPV16 SLP in cervical cancer patients and showed tumor regression in 43% of the patients.[102] Moreover, a randomized clinical trial in recurrent HPV-16 driven cancer confirmed the synergistic effect of the combination HPV-16 vaccination and PD-1 inhibition (median progression-free survival of 2.7 months and median overall survival 17.5 months).[103]

In order to select an appropriate strategy for immunotherapy in VSCC patients, comprehensive studies of the VSCC TME are required. Subsequently, accurate selection of patients that might benefit from immunotherapy can be envisioned.

SCOPE OF THIS THESIS

Before 1980, surgery for all VSCC stages was extensive and consisted of radical vulvectomy with *en-bloc* lymphadenectomy of the groins and enlarged pelvic nodes.[7, 27, 40] Current treatment has evolved into a more conservative and individualized multidisciplinary approach, without compromising prognosis.[7, 27, 28, 40, 41] Despite those treatment adjustments, there is a need to understand tumor behavior and to improve the risk stratification of an individual patient. Identification of molecular markers and risk factors predictive of recurrence risk or death beyond current clinicopathological factors would be a major improvement. This will not only promote accurate prediction of prognosis, but may also lead to tailoring of current and new treatment options.

The first part of this thesis provides insight in prognostic markers in VSCC to refine clinicopathological risk assessment. One of the most frequently described risk factors for recurrent disease is the minimal peripheral surgical margin. In order to improve the quality of future studies and clinical recommendations, we provided a practical guideline on how to uniformly measure this margin in chapter 2. We also determined the clinical relevance of the molecular classification of VSCC based on immunohistochemical staining for p16 and p53. In chapter 3 we described the immunohistochemical characterization of these molecular subtypes to aid their detection in routine clinical practice. We utilized this approach to show the difference in clinical outcome between the three distinct molecular subtypes of VSCC in chapter 4.

The second part of this thesis contains studies on the tumor microenvironment as a first step towards immunotherapy for VSCC. An overview of the literature concerning immunity in VSCC at the start of our studies is provided in chapter 5. Subsequently, we interrogated the TME of different VSCC subtypes in chapter 6, and showed that high infiltration of $CD4^+$

T cells is important for clinical outcome, irrespective of the molecular subtype of VSCC. In chapter 7 we performed an in-depth analysis on the TME based on RNA profiles and showed that highly T cell infiltrated VSCC are potentially eligible candidates for immunotherapy. In chapter 8 we exploited the expression of CD39 by $CD4^+$ and $CD8^+$ T cells as a marker to identify tumor specific T cells. Finally, in chapter 9 the general aspects and relevance of the studies mentioned in this thesis are combined, discussed, and placed in a broader perspective with suggestions for future research.

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