Tissue microarray in prognostic studies on vulva cancer
Fons, G.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 1

Introduction
Introduction

Vulva cancer is a rare disease that mainly affects elderly women. In The Netherlands about 250 new cases are diagnosed each year. The majority of these patients have a squamous cell cancer. Basal cell carcinomas, melanomas, adenocarcinomas, and sarcomas are even more rare. Vulva cancer spreads by direct extension to adjacent structures as vagina, urethra and anus. Lymphatic metastases in the inguinofemoral lymph nodes may occur early in the disease. Spread will usually start to the inguinal lymph nodes, located between Camper’s fascia and the fascia lata. From these superficial groin nodes, the disease will spread to the femoral nodes, located medially from the femoral vein. From the inguinal-femoral nodes the disease may spread to the pelvic nodes. The overall incidence of lymph node metastases is reported to be approximately 30%. Haematogenous spread to distant sites including the lungs, liver and bone occurs late in the course of vulva cancer. Distant metastases are uncommon in patients with one or two positive groin nodes, but are more frequently observed in patients with three or more positive nodes.

The prognosis of patients suffering from a malignant disease is usually expressed as the 5-year survival from the time of diagnosis or onset of treatment. This prognosis is based on biological characteristics of the tumor, its size and spread at diagnosis and the expected effect of treatment.

Cancer staging systems describe kind and extent of the disease at the time of diagnosis and attempt to place patients with a similar prognosis in the same staging group. FIGO (International Federation of Gynecology and Obstetrics) has developed a staging system that stratifies disease specific survival after treatment. In 1988 FIGO revised the staging system for vulva cancer, putting more emphasis on the role of histological assessment of tumor size and lymph node involvement (Table 1. and Table 2.). This surgical pathologic FIGO staging system used since 1988 provides a more accurate predictor of prognosis than the previous more clinically oriented system. This is (partly) explained by the overriding negative influence of lymph node metastases on the period of survival, along with the limitations of clinical investigation to determine node status. In spite of this improvement remaining limitations of the system were recognized soon after its introduction. In the lower stages I and II, for instance, approximately 30% of the patients without lymph node metastases will develop a recurrence, leading to cancer related death in about half of these. Apparently this implies that tumors with similar histological appearance may show widely different biological behavior. Molecular changes at cellular level underlie the development of every malignancy; many of these still unrecognized. Obviously, tumors with aggressive biological behavior will be characterized by more consequential
molecular aberrations. A better understanding of these cellular processes may offer the opportunity to discriminate apparently similar tumors from each other, where presently available systems are unable to do this. Hence our interest in assessing the value of protein markers in vulva cancer.

Stage III comprises a group of patients, which are far from homogeneous: patients with extensive local disease are included as well as patients with a tumor disseminated to lymph nodes on one side. Clearly, prognostic accuracy in vulva cancer is theoretically open to considerable improvement.

Two strategies can be followed to improve prognostic accuracy. First, to assess a possible contribution to prognostic accuracy of protein markers reflecting molecular changes in tumor cells, second, to develop novel clinical pathologic parameters in addition to the ones used in the current staging system.

### Table 1. FIGO stage 1988

<table>
<thead>
<tr>
<th>Stage</th>
<th>TNM Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>T1N0M0 Tumor confined to the vulva or perineum, ≤ 2cm in greatest dimension, nodes are negative</td>
</tr>
<tr>
<td>Stage II</td>
<td>T2N0M0 Tumor confined to the vulva and/or perineum, &gt; 2 cm in greatest dimension, nodes are negative</td>
</tr>
<tr>
<td>Stage III</td>
<td>T3N0M0 Tumor of any size with 1. Adjacent spread to the lower urethra or the anus 2. Unilateral regional lymph node metastases</td>
</tr>
<tr>
<td></td>
<td>T3N1M0</td>
</tr>
<tr>
<td></td>
<td>T1N1M0</td>
</tr>
<tr>
<td></td>
<td>T2N1M0</td>
</tr>
<tr>
<td>State IVA</td>
<td>T1N2M0 Tumor invades any of the following: Upper urethra, bladder mucosa, rectal mucosa, pelvic bone or bilateral regional node metastases</td>
</tr>
<tr>
<td></td>
<td>T2N2M0</td>
</tr>
<tr>
<td></td>
<td>T3N2M0</td>
</tr>
<tr>
<td></td>
<td>T4 any N M0</td>
</tr>
<tr>
<td>Stage IVB</td>
<td>Any T, any N, M1 Any distant metastasis including pelvic lymph nodes</td>
</tr>
</tbody>
</table>

### Table 2. TNM classification

T: Primary Tumor

- T1 Tumor confined to the vulva and/or perineum 2 cm or less in greatest dimension
- T2 Tumor confined to the vulva and/or perineum more than 2 cm in greatest dimension
- T3 Tumor involves any of the following: lower urethra, vagina, anus
- T4 Tumor involves any of the following: bladder mucosa, rectal mucosa, upper urethra, pelvic bone

N: Regional lymph nodes

- N0 No lymph node metastases
- N1 Unilateral regional lymph node metastases
- N2 Bilateral regional lymph node metastases

M: Distant metastases

- M0 No distant metastases
- M1 Distant metastases (pelvic lymph node metastases is M1)
Background

An outline will be given of well-established clinical and histological factors that affect survival of patients with vulva cancer. Second, a description is given of protein markers which may permit the differentiation between tumors with dissimilar behavior. Third, an explanation is given of the new technique of tissue micro array (TMA), developed to enable retrospective antigen expression studies on a larger scale.

Clinical and pathologic prognostic factors

Between 1983 and 2007 sixteen papers were published on this subject.(6-21) In three studies, assessment of prognostic factors was limited to patients with lymph node metastases.(12;13;21) The remaining thirteen papers include data from a total of 2835 patients without or with lymph node metastases. Table 3 lists the various factors and shows the number of authors attributing significant prognostic value to each of these predictors. Some authors performed only a univariate analysis (19;20), others performed both univariate and multivariate analysis.(8;9;11;16;17) In six papers analysis was limited to a multivariate test.(6;7;10;14;15;18)

The summary of the outcomes of multivariate analyses clearly shows the effect of lymph node metastases on the period of survival. The presence of positive lymph nodes was reported as an independent factor limiting survival in 6 out of 7 studies (Table 3.). The number of positive nodes was found to be of independent significance in 4 out of 6 studies. Only Burger et al. and Pinto et al., studied both the existence of lymph node metastases and the number of positive nodes by multivariate analysis. In the study by Burger et al. the number of positive nodes was not a significant indicator for the period of survival after correction for the presence of lymph node metastases. In contrast, Pinto et al. found the number of lymph node metastases to be of independent significance for survival.

In three studies the FIGO stage of the disease was entered into the multivariate analysis. (9;15;16) Only in the study by Chan et al. stage was included in the multivariate analysis together with the presence of more than two lymph node metastases. In this study both parameters were independent prognostic factors for the period of survival.

The diameter of the tumor was the second most important factor for survival (Table 3). In four studies both lymph node metastases and tumor diameter were entered into multivariate analysis.(6-9) In two, tumor diameter was an independent factor for survival after correction for lymph node metastases.(7;9) Only the study by Boyce et al. did not report the existence of lymph node metastases as an independent factor for survival. The mean 5-years disease specific survival rate reported in these studies was 85% (81%-91%)
for patients without lymph node metastases. For patients with lymph node metastases this percentage was only 53% (41%-62%). (7;8;10;15;17)

T-status, qualifying for stage III or stage IV, was included in multivariate analysis in two studies.(11;18) In neither study this factor was of independent prognostic significance.

A recent study on patients with stage III vulva cancer showed that patients with T3 tumors have a significantly better prognosis than patients with stage III disease on the basis of lymph node metastases.(5) This is in accordance with the results of the study by Lataifeh et al.(11)

Only in the study by Homesly et al. the data of patients without lymph node metastases were analyzed separately.(10) In this group of patients the diameter of the tumor was found to have a significant negative influence on the period of survival. In patients with

<table>
<thead>
<tr>
<th>Predictor of survival</th>
<th>References of papers</th>
<th>Number of studies examining the predictor</th>
<th>Number of studies finding the predictor significant in univariate analysis per number of studies</th>
<th>Number of studies finding the predictor significant in multivariate analysis per number of studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>(6;8;10;14;17-20)</td>
<td>8</td>
<td>2/3</td>
<td>0/6</td>
</tr>
<tr>
<td>Clinical N status</td>
<td>(10;19)</td>
<td>2</td>
<td>0/1</td>
<td>1/1</td>
</tr>
<tr>
<td>T status</td>
<td>(11;18;20)</td>
<td>3</td>
<td>1/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Figo stage</td>
<td>(9;15;16;19)</td>
<td>4</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Grade</td>
<td>(6;8;10;11;15;17;19)</td>
<td>7</td>
<td>0/4</td>
<td>1/6</td>
</tr>
<tr>
<td>Location on vulva</td>
<td>(17;19)</td>
<td>2</td>
<td>0/2</td>
<td>0/1</td>
</tr>
<tr>
<td>Clitoral site</td>
<td>(6;19)</td>
<td>2</td>
<td>0/1</td>
<td>1/1</td>
</tr>
<tr>
<td>Diameter of tumour</td>
<td>(6-10;15-17;19;20)</td>
<td>10</td>
<td>3/6</td>
<td>4/7</td>
</tr>
<tr>
<td>Thickness of tumour or depth of invasion</td>
<td>(7-10;14;16-18)</td>
<td>8</td>
<td>2/4</td>
<td>2/7</td>
</tr>
<tr>
<td>Multifocality</td>
<td>(8;16)</td>
<td>2</td>
<td>1/2</td>
<td>0/1</td>
</tr>
<tr>
<td>Surgical margin</td>
<td>(9;10;14;16;18;19)</td>
<td>6</td>
<td>2/3</td>
<td>3/5</td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td>(6-9;11;14;17;19;20)</td>
<td>9</td>
<td>6/6</td>
<td>6/71</td>
</tr>
<tr>
<td>Number of positive nodes</td>
<td>(8;10;14-16;18)</td>
<td>6</td>
<td>1/2</td>
<td>4/6</td>
</tr>
<tr>
<td>Extra-capsular spread</td>
<td>(8;14)</td>
<td>2</td>
<td>0/1</td>
<td>1/2</td>
</tr>
<tr>
<td>Bilateral positive nodes</td>
<td>(8;11;18;19)</td>
<td>4</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td>LVS1</td>
<td>(7-11;16;17;20)</td>
<td>8</td>
<td>6/6</td>
<td>2/7</td>
</tr>
</tbody>
</table>
tumors ≤2 cm in size the relative survival at 5 years was 98%, whereas this figure was 66% in the group of patients with tumors > 8 cm in size.

Studies by Origoni, Paladini and van der Velden et al. included only patients with lymph node metastases. In the studies by Raspagliesi, Pinto and Burger et al. the data of patients with lymph node metastases were analyzed separately. All six authors but one concluded that extra-capsular spread is the most significant independent lymph node associated factor. Only Burger et al. found no statistically significant difference between the overall survival of patients with or without extra-capsular spread of disease. The mean 5-years disease specific survival rate was 74% (51%-87%) for patients with intra-capsular metastases against 27% (15%-41%) for patients with extra-capsular disease.

In conclusion the survival of patients with vulva cancer would appear to be mainly determined by the presence at the time of diagnosis of lymph node metastases and (to a lesser degree) by the diameter of the tumor. Among the node related parameters, extra-capsular spread was found to be strongly associated with impaired survival.

In patients without lymph node metastases, i.e. FIGO stage I and II, tumor diameter has been shown to be the only factor of significance for survival. In spite of the low stage of their initial disease 15% of these patients will eventually die of recurrent vulva cancer. At this moment, this subgroup with a higher risk cannot be identified on the basis of existing clinical pathologic features. In this group of patients additional factors of prognostic significance, such as protein markers, need to be developed in order to improve risk estimation.

Patients with unilateral or bilateral lymph node metastases are assigned to different stages, i.e. stage III and stage IVA, although there is no evidence for a difference in survival. The same applies to patients with T3 or with T4 tumors. The presence of extra-capsular spread is not taken into account in the current staging system. The separation and restaging of patients with current stage III and stage IV disease can be expected to improve discrimination. It can also be recommended to include extra-capsular spread as a prognostic factor into the current staging system.

In general the definition of groups of patients with a similar risk of recurrence or of death by a given type of cancer will be the key to specific medical or surgical treatment aimed at reducing the risk of an adverse outcome. The treatment chosen must of course only be implemented after solid proof of its beneficial effect. This, however, has not always been the case.

An example is the way radiotherapy was introduced into the treatment of patients with vulva cancer. In 1986 a study was published in which patients with positive groin nodes
after radical vulvectomy with bilateral groin node dissection were randomized to receive either radiation therapy on groins and pelvis or pelvic node dissection. The difference in survival of the 114 evaluable patients was significant, favoring the group receiving adjunctive radiotherapy. The advantage was confined to patients with clinical suspicious nodes or more than one positive groin node. Since then patients with more than one lymph node metastasis or with one lymph node metastasis with extra-capsular spread are treated with adjuvant external beam radiation to groins and pelvis. Although the study mentioned did not produce evidence of a beneficial effect of radiation to the pelvis, this treatment was implemented promptly and still is considered standard treatment.

In a similar way the suggestion that patients with one intra-capsular metastasis have a worse prognosis than patients without lymph node metastases led to studies on the beneficial effect of adjuvant radiotherapy in the group of patients with one lymph node metastasis. This suggestion would seem to be supported by the results of a recent study, by Parthasarathy et al. who used data from the SEER (Surveillance, Epidemiology and End Results) database. They reported an improvement of disease specific survival in this group of patients by adjuvant radiation after surgery in patients with one lymph node metastasis. As extra-capsular spread of tumor was not taken into account, these results have to be considered as an insufficient basis for clinical practice. Preferably, this kind of question is answered by performing a prospective randomized controlled trial. When, however, this is impracticable because of the small number of patients fulfilling study criteria, retrospective studies will have to provide the answers. In that case, adjustment must of course be made for all potential confounders.

### Protein markers

In the past decades evidence increased that cancer cells frequently harbor abnormal chromosomes. In 1960 the Philadelphia chromosome was discovered in cells of chronic myelogenous leukemia. This was the first chromosomal anomaly that was specifically implicated in a neoplasm. Proto-oncogenes were identified, encoding for proteins in the cell signaling pathways. When these genes mutate into oncogenes, changing their function, this results in an unregulated stimulation of cell division. There are hundreds of known oncogenes. Tumor suppressor genes have the physiological role of retarding cell division. The gene DNA sequences are made into a protein by the intermediate steps of (1) transcription into RNA, (2) splicing into a much shorter mRNA and (3) translation into protein.

Immunohistochemistry permits the visualization of antigens in tissue sections. Antigens are usually proteins. In immunohistochemistry two major components are used: (1) a primary antibody and (2) a detection system to identify the resulting antigen-
antibody complex. Antibodies react with a part (in case of monoclonal antibodies) or parts (in case of polyclonal antibodies) of antigens. Because an antigen-antibody complex is invisible by conventional light microscopy, a technique must be used which produces a visible product at the site of the antigen-antibody reaction.

Several protein markers have been reported to be of prognostic value in vulva cancer or other squamous cell cancers as head and neck cancer, esophagus cancer or lung cancer. Immunohistochemistry is used to demonstrate these markers. Some of these markers, summarized in Table 4, are briefly described.

Table 4. Antigens

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratin markers</td>
<td></td>
</tr>
<tr>
<td>CK 5/6</td>
<td>c</td>
</tr>
<tr>
<td>CK 10</td>
<td>c</td>
</tr>
<tr>
<td>Proliferation marker</td>
<td></td>
</tr>
<tr>
<td>Ki-67</td>
<td>n</td>
</tr>
<tr>
<td>Tumor suppressor genes</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>n</td>
</tr>
<tr>
<td>p16(^{\text{INK4}})</td>
<td>n/c</td>
</tr>
<tr>
<td>p21</td>
<td>n</td>
</tr>
<tr>
<td>Oncogenes</td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>n</td>
</tr>
<tr>
<td>EGFR</td>
<td>m</td>
</tr>
<tr>
<td>Apoptosis marker</td>
<td></td>
</tr>
<tr>
<td>Caspase-3</td>
<td>c</td>
</tr>
<tr>
<td>Prostaglandin biosynthesis marker</td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>c</td>
</tr>
<tr>
<td>Angiogenesis marker</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>c/m</td>
</tr>
</tbody>
</table>

c indicates cytoplasm; m, membrane; n, nucleus

Progression through the cell cycle is governed by a family of cyclin-dependent kinases (cdks). Activity is regulated by phosphorylation, activated by binding of cyclins and inhibited by cdk inhibitors. The cdks regulate biochemical pathways or checkpoints that integrate mitogenic and growth inhibitory signals, monitor chromosome integrity, and coordinate the precise timing of cell cycle transitions.\(^{28}\)

The pRb pathway is mediated by functional inactivation of Rb through its phosphorylation by a complex of cyclin-D1 and Cdk4 or Cdk6 and subsequent release of E2F.
Two families of cdk inhibitors negatively regulate cdk activities and mediate cell cycle arrest following growth inhibitory stimuli. P16\textsuperscript{INK4} is a member of the INK4 (inhibitor of cdk4) family. P21\textsuperscript{WAF/CIP1} (p21) is a member of the kinase inhibitor protein (KIP) family. The p16\textsuperscript{INK4} protein may act as a cyclin-dependent kinase inhibitor by binding competitively to Cdk4, thus preventing the interaction Cdk4-Cyclin D1. Control of cell cycling by this pathway may be lost in virtually all tumors, either through disrupted p16\textsuperscript{INK4} function or mutations of Rb.

Loss of p16\textsuperscript{INK4} occurs by independent mechanisms, namely deletions, point mutations, or promoter hypermethylation. P16\textsuperscript{INK4} is currently considered to be the most frequently inactivated tumor-suppressor gene in cancer.

P21 is a transcriptional target of p53 and is involved in senescence and differentiation. The Cip/Kip family inhibits the cdk2-complexes, but also acts as positive regulator of the Cyclin D-dependent kinases by increasing their stability and directing the Cyclin D/cdk complex to the nucleus. In a variety of human malignancies, an association has been observed between aberrant expression of p16\textsuperscript{INK4} and p21, and an unfavorable prognosis.

Expressions of p16\textsuperscript{INK4} and retinoblastoma protein were measured in 32 cases of squamous cell cancer of the vulva. (29) Sixty-two percent of the tumors did not show p16\textsuperscript{INK4} expression. These data are in agreement with those from a more recent study on p16\textsuperscript{INK4} and p21 expression in 151 vulva carcinomas by Knopp et al.(30) This study reported an association between absent p16\textsuperscript{INK4} staining and lymph node metastases in univariate analysis. In head and neck- and oropharynx cancer, absent p16\textsuperscript{INK4} expression was significantly associated with shorter period of survival.(31;32)

In the study by Knopp et al. p21 expression was assessed as well. Eighty-six percent of the tumors showed p21 expression in less than 10% of the cells. There was no significant association between overall survival and p21 expression.

Cyclin D1 expression was measured in 57 vulva cancers by Rolfe et al.(33) Fifty-one percent of tumors showed over-expression (defined as >10% of cells), no association with the FIGO stage was found. This percentage of over-expression is considerably higher than the 21% positive staining (defined as >10% of cells) found in a study on 38 vulva cancers by Lerma et al.(34) An association between Cyclin D1 expression and prognosis was not mentioned in both studies. In a study on biomarkers in squamous cell carcinoma of the tongue, patients with Cyclin D1 positive tumors (defined as > 10% of cells positive) had a significantly shorter period of overall survival than patients with Cyclin D1 negative tumors (RR 1.86, 95% CI 1.01 - 3.44, p=0.025).
**p53**
The p53 tumor suppressor gene is involved in the regulation of the cell cycle and the triggering of apoptosis. Mutations in the p53 gene are found in 50% of human cancers. A study on p53 expression in vulva cancer did not find an association between p53 and lymph node metastases.\(^{(35)}\) Likewise, a study on p53 expression in 66 vulva cancers reported no significant association between p53 over-expression (defined as >10% of cells positive) and lymph node metastases or survival.\(^{(36)}\) These data are not in agreement with those from a study by Scheistroen et al. which reported a p53 over-expression (defined as >5% of cells positive) in 55% of the cases of squamous cell cancer of the vulva.\(^{(37)}\) Patients with p53 over-expression had a significantly worse prognosis compared with patients with p53 negative tumors (\(p=0.039\)).

**Ki-67**
Ki-67 is a non-specific nuclear marker for proliferation, which has been shown to be a prognostic factor in numerous types of cancer, including several gynecologic tumors. In normal vulva tissue, Ki-67 expression has been found in the basal and para-basal cells of the squamous epithelium. In a study on 31 vulva tumors a diffuse staining pattern did correlate with poor differentiation (RR 3.59, 95% CI 1.59-7.60, \(p=0.013\)) but not with lymph node metastases.\(^{(38)}\) The association with survival was not studied.

**Cytokeratin 5/6 and 10**
Human cytokeratins (CK) constitute a family of 20 distinct polypeptides, which are differentially expressed in various epithelia at successive stages of differentiation and development. Cytokeratin 5/6 is mainly found in the basal layer of normal epithelium. CK10 is expressed in all suprabasal layers. A close relationship exists between expression of the cytokeratins and grade. Cytokeratin 5/6 has not been shown to be of prognostic value in vulva cancer in the literature. Expression of CK10 was studied in samples of 41 vulva cancers. No relationship was found between expression of CK10 and tumor recurrence.\(^{(39)}\)

**Caspase-3**
A recently discovered family of proteases, called caspases, is the key effector of cellular death. Among the caspases, caspase-3 would appear to correlate best with apoptosis.\(^{(40)}\) At the start of our studies no data were available on the prognostic significance of caspase-3 expression in vulva cancer. A study in non-small cell lung cancer reported a significantly longer median period of survival for patients with caspase-3 positive carcinomas than for those with caspase-3 negative tumors.\(^{(41)}\) A significantly lower incidence of lymph node metastases was found in the caspase-3 positive group. Similar
results were obtained in a study on the prognostic significance of caspase-3 expression in primarily resected esophageal squamous cell carcinoma.\(^{(42)}\)

**EGFR**

The epidermal growth factor receptor (EGFR) is one of four homologous transmembrane proteins that mediate the actions of a family of growth factors including EGF, transforming growth factor-alfa, and the neuregulins.\(^{(43)}\) Abnormal expression and/or mutation of the EGFR have been implicated in the progression of some solid tumors. In a study on 61 vulva cancer patients EGFR expression was evaluated in neoplastic and non-neoplastic tissue from the same patient.\(^{(44)}\) A progressive increase in EGFR expression was shown from benign vulva epithelium to primary malignant tissue to metastatic lesions. Increased expression in the primary tumor (defined as >90% of cells) was significantly associated with lymph node metastases \((p<0.025)\) and decreased 5-years survival \((25\% \text{ vs. } 54\%, p<0.05)\). In other tumors data on EGFR expression and outcome are conflicting. Extensive research is done on the relationship between EGFR expression and outcome in non-small cell cancer of the lung (NSCLC). In a meta-analysis published in 2001, 16 studies on EGFR expression in NSCLC were evaluated.\(^{(45)}\) One trial reported a survival benefit of EGFR expression, three a survival disadvantage and twelve no statistically significant difference. The result of the meta-analysis on eight studies using immunohistochemistry showed a weak survival benefit for patients with EGFR-negative tumors \((HR \ 1.13, \ 95\% \ CI \ 1.00 \ to \ 1.28)\).

**VEGF**

Angiogenesis is an essential component of solid tumor growth and metastasis.\(^{(46)}\) Vascular endothelial growth factor (VEGF) is a cytokine that promotes vascularisation in the tumor by activating the host endothelium.\(^{(47)}\) The influence of VEGF expression on prognosis in vulva cancer was studied in 25 patients with squamous cell cancer.\(^{(48)}\) A survival benefit was found in the group of patients with tumors with poor VEGF expression \((10 \text{ year overall survival } 86\% \text{ vs. } 24\%, \log \text{ rank } p<0.01)\).

**Cyclooxygenase-2**

Cyclooxygenase (COX) catalyses the synthesis of prostaglandin’s (PG) from arachidonic acid. Two enzyme isoforms have been identified: COX-1 which is expressed as a ‘housekeeping’ gene in most of the cells and COX-2 which is expressed as an early-response gene activated by many stimuli such as inflammatory cytokines as well as growth factors, and oncogenes.\(^{(49)}\) Expression of COX-2 has been studied in many different tumors. In general over-expression of COX-2 in squamous cell cancer is associated with poor
In a study on COX-2 expression in neoplastic vulva epithelial lesions, COX-2 over-expression was significantly associated with lymph node metastases. (53)

**Tissue Micro Array**

Tissue micro array (TMA) allows the assessment of hundreds of tissue samples from a larger group of patients on a single slide by using immunohistochemistry, fluorescence in situ hybridization or RNA in situ hybridization. (54) The construction of a TMA comprises the following steps. One or 2 representative hematoxylin and eosin slides are selected of each tumor. Usually, three areas of interest are encircled on each slide. In the corresponding paraffin donor block 0.6 mm cores are punched out. These cores, each 3-4 mm high are then embedded in the recipient block using a manually operated TMA device. The spacing between the cores is 1 mm. The recipient block is sectioned at 4 μm, and the sections are transferred to glass slides. One slide can contain tumor cores of 40 to 50 patients. Every slide contains some negative and positive control cores. The slides are immunoassayed with a panel of antibodies against various proteins, depending on the research question. A major concern is the extent to which individual tumor heterogeneity may affect the validity of the results. Although studies on gastric cancer, bladder cancer and breast cancer show that findings from routine sections can be reproduced in TMA (55-57), the validity of this method has not yet been tested in vulva cancer. The TMA technique guarantees uniform staining of all tissues. The subjective element in the scoring of immunohistochemical results has not been excluded by this technique. Cut-off points, as determined in a studied population, may not be appropriate in other independent populations. The validity of results of immunohistochemical tests performed with TMA has to be determined in two different ways. First, a comparison between test results on TMA and whole slides has to be made. Second, an external validation has to be performed by assessing the reproducibility of an association between expression patterns and period of survival in an independent but similar study group.
Aims of the studies

The aim of the studies, described in this thesis, was to explore methods to improve prognostic accuracy in vulva cancer patients, both by the assessment of relatively novel protein markers and the re-examination of well-established clinical pathologic parameters.

As extra-capsular spread was identified early in our studies as an important negative prognostic factor, we analyzed separately the beneficial effect of adjuvant radiotherapy for patients with one lymph node metastasis but without extra-capsular spread.

The more specific aims of each study are summarized in table 5.

**Table 5. Specific aims of studies**

<table>
<thead>
<tr>
<th>Aim</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td>to identify immunohistochemical markers with prognostic significance for disease specific survival with a test TMA</td>
<td>2</td>
</tr>
<tr>
<td>to validate TMA for vulva cancer by comparing immunohistochemical staining results of triplicate core biopsies on TMA with the results of full section analysis</td>
<td>3</td>
</tr>
<tr>
<td>to validate the results of the test TMA showing that COX-2 over expression and absent Caspase 3 expression are associated with poor disease-specific survival</td>
<td>4</td>
</tr>
<tr>
<td>to assess the reproducibility of the association between over expression of COX-2 and survival in an independent but similar study group of vulva cancer patients</td>
<td>5</td>
</tr>
<tr>
<td>to determine whether laterality of lymph node metastases has prognostic significance, independent of the number of lymph node metastases and to determine the prognostic significance of extra-capsular spread irrespective of the number of lymph node metastases</td>
<td>6</td>
</tr>
<tr>
<td>to analyze the benefit from adjuvant radiotherapy in patients with vulva cancer and a single positive node without extra-capsular spread.</td>
<td>7</td>
</tr>
</tbody>
</table>
References


