Carcinogenesis and tumor progression of squamos cell carcinoma of the vulva
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Clinical and histopathological aspects of vulvar carcinoma

This thesis starts with the clinical impact of prognostic factors and consequences of treatment to patients with vulvar carcinoma. To illustrate the problem here we should go back into the history of the treatment of patients presenting with vulvar carcinoma. In the early part of this century standard treatment of vulvar cancer comprised radical vulvectomy with bilateral inguino-femoral lymphadenectomy and pelvic lymphadenectomy. This radical surgical approach markedly increased the survival rates, but postoperative morbidity was high. Fortunately, a change was made towards more individualized surgical treatment of patients in the management of vulvar cancer during the eighties. Major steps in this more tailored treatment were: vulva conserving operations, elimination of pelvic lymphadenectomy, omission of groin node dissection for patients with stage I tumors with less than 1 mm of stromal invasion, unilateral groin node dissection and use of separate incisions for groin node dissection. Nowadays, for patients with T1N0-1 vulvar carcinoma wide local excision instead of vulvectomy is advocated, depending on the conditions of the vulva of the patients. Still, dissection of groin nodes remains to be standardly performed when stromal invasion is more than 1 mm. The issue here is that only a small group benefits of this approach, because most vulvar carcinoma patients, attending a physician, present with a tumor that already exceeds 1 mm of stromal invasion. So, the starting point here was to look for clinico-pathologic parameters with which lymph node metastases could be predicted in order to benefit the majority of the group of patients with vulvar cancer without negatively affecting survival. Multivariant analysis was performed on the routinely used histopathologic parameters in order to evaluate their value to predict lymph node metastases. Depth of invasion appeared to be the most predictive parameter of lymph node metastases followed by differentiation grade. However, even in cases with 1-3 mm invasion and a well differentiated tumor, the point estimate of the risk for lymph node metastases was 9%. This probability was too high to refrain from lymphadenectomy. At this point it was clear that we had to look for other parameters that could improve our insight in tumor behaviour of squamous cell carcinoma of the vulva.
Proliferation markers

Cell proliferation is an essential part of growth processes and certain proteins are specifically expressed during proliferation. These are called tumor markers and can be used to assess proliferation. In our study the choice of proliferation markers was based on certain criteria:

1. the proliferation markers had to be detectable in formalin-fixed paraffin-embedded material
2. cells expressing the protein had to be markedly positive to enable appropriate quantitation
3. an easy and inexpensive detection method was desired.

This had led to our choice of Ki-67, which is a protein expressed during the cell cycle except for the G0-early G1 phase, and Ag-NOR, which are nucleolar organizer regions involved in cell proliferation. As a reference we used mitotic index. The finding that Ki-67 index, but not Ag-NOR count was related to mitotic index has raised questions about the usefulness of Ag-NOR as a proliferation marker.

In a couple of studies the results of flow cytometry and BrdU-labelling were correlated with Ag-NOR staining. It was demonstrated that pAg-NOR (percentage of tumor cells that harbor more than 5 Ag-NORs per cell) correlates with percentage of cells being in S-phase (proliferative activity), whereas mAg-NOR (mean number of Ag-NOR dots per cell or nucleus) correlates with amount of DNA inside the cell (ploidy) (7,9,10). This could be an explanation why Ag-NOR staining in our study did not correlate with mitotic index. Nonetheless, we found no relation between proliferation and lymph node metastases, which indicates that excessive cell proliferation in vulvar carcinoma does not reflect tumor progression. In the context of the carcinogenesis model of J. Miller and E. Miller (5), it may be assumed that excessive proliferation of tumor cells reflects tumor promotion rather than tumor progression.

At the 88th annual meeting of the AACR (April 1997) it was postulated by Kerbel that though it is hardly disputable that oncogenes do have impact on stimulating the aberrant proliferation of tumor cells harboring such mutations, their impact on tumor growth and development - including that of distant metastases - has probably been overestimated for many types of solid tumor such
as breast and prostate cancer. The two novel generic functions of oncogenes involved in the development and spread of solid tumors, as proposed by Kerbel, are stimulation or facilitation of angiogenesis and enhancement of tumor cell survival. Rak et al (13) have demonstrated that mutant ras oncogenes upregulate VEGF/VPF expression and in another study Rak et al (14) showed that massive apoptotic death effect in rat intestinal epithelial cells can be aborted by constitutive or transient expression of a mutant ras oncogene. With respect to metastasis, oncogenes can also induce or upregulate molecules thought to be involved in cell motility, migration, invasion and adhesion (i.e. proteases and adhesion molecules). It follows that the contribution of oncogenes to tumor progression may reside in stimulating angiogenesis and cell survival (as well as cell invasion) rather than in their direct contribution to inducing aberrant cell proliferation.

**DNA flow cytometry and tumor ploidy**

Measurement of DNA content is a useful method to determine genomic status of tumor cell populations, but comparison of obtained data is hampered by discrepancies between the interpretations of DNA histograms. Particularly in paraffin-embedded material misinterpretation of DNA histograms is caused by the fact that an admixture of tumor and non-tumor cells is measured. Small deviations in DNA contents of tumor cells are not easily detected or even remain undetected. In epithelial tumors anti-cytokeratin labeling enables discrimination between tumor and non-tumor cells, reducing the chance of misinterpretation of the DNA histogram of the tumor cell population. We related sidescatter characteristics of the tumor population to keratin labeling and found these parameters to be highly correlated. Gating on sidescatter characteristics is easy to perform and no additional techniques are required. This simple modification appeared to be a useful tool in distinguishing tumor cells from non-tumor cells. Moreover, by gating on sidescatter we revealed near diploid and multiploid tumor cell populations which go undetected by conventional DNA flow cytometric analysis. Though we could not establish the clinical relevance of this modified method, the percentage metastasized tumors in the group of tetraploid and multiploid tumors was higher than in the groups of diploid and aneuploid tumors.
This could indicate that polyplodisation in tumors reflects a final stage of tumor progression. Recently, p53 has been implicated in a G2/M phase checkpoint. This p53-mediated G2/M checkpoint may account for genomic instability that is commonly associated with p53 mutation (2). A possible route could be: initiation of diploid cells leads to promotion, cell division gets disturbed, polyplodisation occurs aneuploid cell populations will emerge due to chromosomal loss. Fukasawa et al. (4) demonstrated that mouse embryo fibroblast of p53 null mice produce abnormal numbers of centrosomes in a cell (not observed in normal cells) after a few doublings in cell culture and initiate spindles with three or four poles. These p53 null cells rapidly become aneuploid at times when cells with wildtype p53 remain diploid during their passages.

**Tumor suppressor genes, proto-oncogenes and HPV**

We investigated interrelations between the tumorsuppressor genes p53 and Rb and proto-oncogenes mdm2 and p21, and the impact of HPV on the expression of these genes. Overexpression of p53 was found to be a late event, occurring markedly in primary invasive vulvar tumors and their metastases. This indicates that carcinogenesis of SCC of the vulva is a multistep process in which mutations of p53 occur late in the phase of tumor progression being closely related to metastatic potential of the primary tumor. Two inhibitors of angiogenesis are angiostatin and thrombospondin-1 of which the latter is regulated by wildtype p53. Mutation or loss of wildtype p53 is associated with loss of TSP-I production and a shift in the balance towards stimulation of angiogenesis (3). In this respect it is worth mentioning that tumor angiogenesis is highly associated with metastasis and tumor dormancy (11).

Inhibition of p53 functioning is not likely to be due to mdm2 amplification as our data showed a decline of mdm2 expression with severity of the lesion. A possible explanation for the decline of mdm2 is that mdm2 is not upregulated by aberrantly expressed p53. P53 dysfunctioning could neither be ascribed to HPV interference, because less than 10% of the cases investigated were HPV (-16, -18, -31, -33) positive. Of these 10 HPV positive cases 4 showed expression of p53. If overexpression of p53 found in this series of vulvar carcinomas is related
to mutations in the p53 gene, then these mutations might have been arisen spontaneously.

In benign lesions HPV DNA exists as an extachromosomal plasmid. In many cancers HPV DNA is integrated into the host chromosome. Integration of the viral genome, occurring in the E1/E2 region, frequently disrupts the E2 ORF, which encodes transcription regulatory proteins. Loss of these proteins leads to dysregulation of E6 and E7 expression (16). Moreover, by splicing out an intron within E6, an E6* transcript is generated, which appears to be specific for HPV's related to cervical carcinoma. In the oncogenic HPV the E7 protein may be encoded by the E6*E7 transcript, whereas in the non-oncogenic virus type two separate transcripts are generated: one for E6 and one for E7 (15). In HPV 6 and 11, the non-oncogenic types, there is no evidence for E6* splicing. Detection of E6 and E7 transcripts is associated with integration of HPV DNA into the host chromosome, whereas detection of E1 and E2/E4/E5 and the late genes (L1 and L2) indicates productive HPV infection. Böhm et al (1), found expression of E4/E5 in two of four vulvar carcinomas and expression of L1 only in one case. He concluded that in the cases not expressing E4/E5, HPV DNA was integrated in the host chromosome. Park et al. (12) found transcripts of the E6-E7 region to be more abundant than those of the L1-L2 region in all four lesions. Viral capsid antigen was detected in a few well-differentiated cells of a warty carcinoma.

HPV infection might, in first instance, contribute to tumor promotion, but depending on how sequences of the viral DNA (especially the genes encoding for E6 and E7) are integrated into the host cell genome, this could lead to tumor progression. In this may lie the clue why HPV is highly associated with cervical cancer and not with vulvar cancer.

The 10 HPV positive cases showed expression of Rb in only one case, which is in concordance with the general theory about HPV E7 interference with Rb. Expression of Rb was not related to expression of p53. Overexpression of Rb may reflect dysregulation of the cell cycle, but the exact route and total impact are not clear. p21, a mediator of p53, was also studied, but no relation was found between p21 and p53 or Rb. It seems that in vulvar carcinomas expression of p21 is p53 independent. The finding that expression of Rb and p21 is related to proliferation, but expression of p53 is not, supports the idea that p53 is involved in the process of tumor progression rather than tumor promotion.
Though the variables studied here do not contribute to a better prediction of lymph node metastases, we gained better insight in carcinogenesis and tumor progression of squamous cell carcinoma of the vulva.

**Conclusive remarks and future aspects**

The major conclusions that can be drawn from this study are:
- neither HPV infection nor excessive proliferation are relevantly involved in metastasis of vulvar cancer
- prevalence of HPV infection may be influenced by the patient population studied
- p53 plays a crucial role in tumor progression of vulvar cancer
- mdm2 is not upregulated by aberrantly expressed p53 in vulvar cancer
- p21 and Rb can be expressed independently of p53 expression in vulvar cancer.

This thesis covers a small part of the total field of carcinogenesis of squamous cell carcinoma of the vulva. Prediction of lymph node metastases does not appear to be easy, illustrating the complexity of this multifactorial event. We acquired more insight about carcinogenesis in general and squamous cell carcinoma of the vulva in particular, which may contribute to future research in this field.

**Etiology.** The field of interest to many investigators of vulvar cancer was the prevalence of HPV infection possibly establishing a segregation in a HPV and a non-HPV related etiology of vulvar cancer. This is a quite acceptable approach, considering the major role HPV infection plays in cervical cancer. A more fundamental question here would be why HPV is not as highly related to vulvar cancer as it is to cervical cancer. Somehow epithelial cells of the cervix seem to be more vulnerable to incorporation of HPV DNA into the host chromosome than epithelial cells of the vulva. A study on the prevalence and expression of HPV genes in normal tissue and sequential lesions like VIN (I-III), invasive carcinoma and metastasizing carcinoma may clarify the genetic basis of the development of different lesions.
However, even if HPV infection would be a significant causative factor in the development of vulvar cancer, the cause of the majority of vulvar cancers, in particular the keratinizing squamous cell carcinomas, still remain unexplained. As the latter group occurs mostly in elderly women, it is conceivable that acquired genetic changes are the main cause of vulvar cancer. In this respect more emphasis should be placed on karyotyping of these tumors. Due to the relative low prevalence and the genetic complexity of these tumors, cytogenetic reports considering this tumor are few.

**Model of carcinogenesis.** The findings in this study regarding regulatory genes at the protein level does not seem to fit in the actual model of cell cycle regulation. Most studies contributing to the model are done on translational level and synchronization of the results should be pursued, before drawing general conclusions. In this respect p53 mutation analysis at the DNA level of larger series of vulvar carcinomas is required for proper interpretation of p53 protein expression. Dysfunctioning of the negative regulators of the cell cycle, like p53, Rb and p21, will affect expression and functioning of the positive regulators, like cyclins and cdk's which can be easily studied and mapped. Abnormalities such as amplification and rearrangement of cyclin D1 have been found in vulvar carcinoma and cervical cell lines (8). Studies on these genes should be extended and related to angiogenesis and tumor cell survival as there are indications that dysregulation of cell cycle genes may facilitates tumor progression rather than inducing it.

**Advances in detection techniques.** The starting point of this study was the need in the clinic for non-invasive or minimal invasive techniques to predict lymph node metastases in squamous cell carcinoma of the vulva. Recently a very elegant study has been done on 10 patients based on detection of the sentinel lymph node (6). This minimal invasive method, earlier described for cutaneous melanoma and breast cancer, involves imaging by radioactive labeling of the sentinel node, the first node in the lymphatic basin that receives primary lymphatic flow. The results are very promising as histopathology of the sentinel node appears to be representative for the presence of lymph node metastases. Further development of this technique may in the near future spare patients with a negative sentinel node full groin node dissection.