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VAGINAL CARCINOMA

STUDIES ON ETIOLOGY AND PROGNOSTIC FACTORS

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ABSTRACT

Primary carcinoma of the vagina (PCV) is a rare disease, with poor survival, mostly affecting elderly women. Due to its rarity the established knowledge originates from studies based on small materials, therefore little is known regarding the genesis, natural history and factors predicting the prognosis. PCV is supposed to have similar etiology and natural history as cervical carcinoma, which however mostly occurs at younger ages. Clinical stage is described as the most important prognostic factor. Against this background the objectives of this thesis were focused on the understanding of the genesis of PCV and its prognostic factors.

The material in the retrospective studies (paper I and II) is the largest so far published on PCV.

A hypothesis was that factors influencing the age at onset of PCV should be connected directly or indirectly with the genesis of the disease. Epidemiological, clinical and histopathological variables were evaluated in relation to **age at diagnosis** in a retrospective study of 341 cases of PCV, diagnosed 1956 – 1996. According to the statistical analysis the independent predictor for young age at diagnosis was a history of cervical dysplasia and for old age at diagnosis late menarche. Parity ≥4 as well as nulliparity, smoking and unstable marital status were background factors more common than in the general Swedish female population, but not correlated with age at diagnosis.

Clinical and histopathological **prognostic factors** were evaluated retrospectively in 314 patients with squamous cell carcinoma of the vagina. The 5-year disease-specific survival rate was 45 % and in stage I 75%. In the multivariate analysis there were only three factors that independently could predict poor survival: high age at diagnosis, large tumours (≥4cm) and advanced stage. Common background factors with no prognostic significance were prior hysterectomy, other gynecological malignancies and pelvic irradiation.

The immunohistochemical expression of **laminin-5γ2 chain**, an epithelial basement membrane protein, implicated in tumour cell invasion, was investigated in 59 cases of primary vaginal malignancies. All epithelial tumours showed $\gamma 2$ chain immunoreactivity. High expression of the $\gamma 2$ chain was a good predictor of poor survival in the univariate analysis, but not in the multivariate analysis. A positive correlation between tumour size and $\gamma 2$ chain expression could also be observed.

Comparative genomic hybridization (CGH) of 16 primary vaginal carcinomas revealed that 70% carry relative copy number increases that map to chromosome arm 3q. The pattern of genomic imbalances was strikingly similar to the one observed in advanced cervical tumours. Most PCV were aneuploid, showed high proliferative activity, low p53 and increased p21 expression. Human papilloma virus was detected in 2/8 cases. In the univariate analysis age at diagnosis, tumour size, and laminin-5 expression were identified as predictors of prognosis.

Protein expression patterns in six samples from primary vaginal cancers, in five from normal vaginal tissue and in five primary cervical cancers, were analyzed using two-dimensional polyacrylamide gel electrophoresis (2-DE). A total of 23 protein spots were significantly differentially expressed among cervical and vaginal carcinoma. By using these 23 proteins in cluster analysis all samples could be classified into three distinct groups (normal vaginal tissue, vaginal- and cervical carcinoma). There might be a possibility to identify tumour-specific markers among the differentially expressed proteins.

In conclusion this thesis supports the theory of related etiology and tumourigenesis for vaginal and cervical carcinoma. Crude clinical variables (stage, tumour size and age at diagnosis) turned out to be independent predictors of prognosis. PCV appears to be an aggressive tumour characterized by high genomic instability, aneuploidy and high proliferative activity.

LIST OF PUBLICATIONS

I. Hellman K, Silfverswärd C, Nilsson B, Hellström AC, Frankendal B, Pettersson F. Primary carcinoma of the vagina. Factors influencing the age at diagnosis. The Radiumhemmet series 1956 – 1996. Int J Gynecol Cancer 2004, 14, 491-501.

II. Hellman K, Lundell M, Silfverswärd C, Nilsson B, Hellström AC, Frankendal B. Clinical and histopathological factors in relation to prognosis in primary squamous cell carcinoma of the vagina. Accepted 2005 Int J Gynecol Cancer.

III. Hellman K, Hellström AC, Silfverswärd C, Salo S, Aspenblad U, Nilsson B, Frankendal B, Tryggvasson K, Auer G. Cancer of the vagina: Laminin-5 gamma2 chain expression and prognosis. Int J Gynecol Cancer 2000, 10, 391-396.

IV. Habermann JK, Hellman K, Freitag S, Heselmeyer-Haddad K, Hellström AC, Shah K, Auer G and Ried T. A recurrent gain of chromosome arm 3q in primary squamous carcinoma of the vagina. Cancer Genetics and Cytogenetics 148 (2004) 7-13.

V. Hellman K, Alaiya AA, Schedvins K, Steinberg W, Hellström AC, Auer G. Protein expression patterns in primary carcinoma of the vagina. British Journal of Cancer (2004) 91, 319-326.

LIST OF ABBREVIATIONS

ANCA	Average number of chromosomal aberrations (=the ratio of all copy number changes divided by the number of cases)
ATM	Ataxia Telangiectasia Mutated (defects in the gene result in AT)
BM	Basement Membrane
BT	Brachytherapy
HDR	High dose rate
LDR	Low dose rate
CA	Correspondence Analysis
CDK	Cyclin Dependent Kinase
CGH	Comparative Genomic Hybridization
CIN	Cervical Intraepithelial Neoplasia
CIS	Carcinoma In Situ
CKI	CDK Inhibitor
2-D PAGE	Two Dimensional Polyacrylamide Gel Electrophoresis
Da	dalton
DES	Diethylstilbestrol
DFS	Disease Free Survival
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
ERT	External Radiation Therapy
ExPASy	Expert Protein Analysis System
FCM	Flow Cytometry
FIGO	International Federation of Gynaecology and Obstetrics
GST	Glutathione-S-Transferase
Gy	gray
HE	Haematoxylin and Eosin
HPV	Human Papilloma Virus
HSP	Heat Shock Proteins
ICM	Image Cytometry
IEF	Isoelectric focusing
IHC	Immunohistochemistry
IPG	Immobilised pH Gradient
kVh	kilovolthour
Ln	Laminin
MALDI-TOF	Matrix-assisted Laser Desorption Ionisation -Time of flight
Mascot	Software program available on line for proteinidentification (www.matrixscience.com)
Menstruation span	Age at menopause minus age at menarche minus the number of births multiplied by 1.5 (in order to take into account the amenorrhea during child-bearing and lactation)
MMP	Matrix Metalloproteinase
MS	Mass Spectrometry
p16, p21, p27, p53	Nuclear phosphoprotein, 16, 21, 27, 53 kDa

Pap smear	Papanicolau smear discovered for detection of dysplasia.
PCNA	Proliferating Cell Nuclear Antigen
PCV	Primary Carcinoma of the Vagina
PDGF	Platelet Derived Growth Factor
PTEN	Phosphatase and tensin homolog deleted on chromosome Ten
Rb	Retinoblastoma gene
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulphate
TERC	The telomerase RNA component
VAIN	Vaginal Intraepithelial Neoplasia
VEGF	Vascular Endothelial Growth Factor
VIN	Vulvar Intraepithelial Neoplasia

CONTENTS

INTRODUCTION

Primary carcinoma of the vagina is a rare disease, representing only 1% of all female genital malignancies in Sweden 2003 [1]. Due to the close proximity of the vagina and the surrounding organs (cervix, uterus, vulva, rectum, and bladder), 80-90% of the vaginal malignancies are secondary tumours originating from these sites.

The age distribution of PCV is characterised by a peak incidence in the old age-group (60-80 year). Annually there are about 35 cases reported to the National Swedish Cancer Registry [1]. The incidence rate of PCV has remained constant for the last decades [2].

The survival rate has increased with 15% over the last 30 years, which primarily is due to both improvements in the irradiation techniques and earlier detection [3]. However, the 5-year survival rate still is quite poor limited to approximately 47%, actuarial, and 63% in stage I [4].

Given the rarity of PCV the established knowledge is based on accumulated experience from different institutions worldwide over a long period of time. Little is known concerning etiologic factors and there are contradictory data regarding clinical and histopathological prognostic variables. The etiology may to be similar to that of cervical carcinoma and clinical stage is considered as the principal variable predicting survival. Biological prognostic factors are rarely studied.

BACKGROUND

HISTORY

Primary carcinoma of the vagina was first described in 1826 by Cruveilhier for the Anatomical Society of Paris [5]. Until 1890 only 57 cases of this disease were described in the literature [6, 7] and there was no effective treatment. Surgical extirpation of vaginal carcinoma was described in the 1890s [6, 8] but at the same time another author stated that any operative treatment was useless [9]. In 1900 the Wertheim surgery for cervical carcinoma was introduced to vaginal carcinoma by Ernst Wertheim [10]. Three case reports on the Wertheim procedure were found in the literature in 1923 but the follow-ups were short [11-13]. For more advanced stages exenterative surgery was described by Alexander Brunschwig in 1948 which implied the vaginal carcinoma was removed together with complete excision of the pelvic viscera. However, the perioperative mortality and morbidity were high [14].

Due to the discouraging results from the surgical treatment and with regard to the close proximity of the pelvic organs to the vagina, radiotherapy came to play the predominant role in the treatment. Radium therapy for PCV patients was first reported in the literature by Taussig in 1929 [15] where only 2 out of 18 patients treated with radium survived for more than 5 years (Table 1). He stated that "primary cancer of the vagina is very rare and almost universally fatal and "we acknowledge our total inability to do anything effective".

By combining radium with deep x-ray therapy the cure rates increased gradually (Table 1). In 1952 Messelt reported of 78 patients treated at The Norwegian Radium Hospital where the five year cure rate was 23% [16] (Table 1). Murphy concluded in 1957 that "x-ray therapy plays a very important part in the radiologic management of primary vaginal cancer" and "more efforts must be directed toward improving the radiologic technique for the advanced lesions" [17].

Table 1. Review of survival rates in published series of PCV.

Author	Study period	Number of patients	Number of 5-year survivors	Overall 5-year survival (%)
Taussig	1906-1929	27	2	7.4
Moench	1915-1930	37	3	8.0
Masson	1910-1927	80	18	22.5
Messelt	1932-1945	78	17	23.0
Palmer	1919-1952	75	24	32.0
Murphy and Bozzini	1919-1955	161	43	27.0
Whelton and Kottmeier	1930-1955	117	31	26.5
Rutledge	1941-1961	43	15	35.0
Benedet	1950-1980	75	34	45.0
Stock	1962-1992	100	48	48.0
Kirkbride	1974-1989	153	89	58.0
Chyle	1953-1991	301	180	60.0
Frank	1970-2000	193	112	58.0

ANATOMY

The vagina is a collapsed tube consisting of mucous membrane and muscle. It extends from the uterine cervix to the vestibule of the vagina and lies anterior to the rectum and posterior to the urinary bladder and urethra. The recess between the cervix and the vagina is called the vaginal fornix. The upper border of the posterior wall of the vagina (1-2 cm) is usually covered by peritoneum and separated from the rectum by the rectouterine pouch (the pouch of Douglas). The perivaginal adventitious connective tissue (paracolpium) connects the vagina with neighbouring organs, a firm connection with the wall of the urethra and a loose one with the wall of rectum (the rectovaginal septum). The vagina opens into the vestibule formed by the urogenital sinus [18].

Blood and nerve supply

The blood supply of the vagina derives from branches of the internal iliac arteries, the vaginal artery, the vaginal branches of the uterine artery, the middle rectal artery and the internal pudendal artery. The venous drainage of the vagina comes from the vaginal venous plexus along the sides of the vagina emptying into the internal iliac veins [19, 20].

The nervous supply of the superior part of the vagina derives from vaginal branches from the uterovaginal plexus which lies in the broad ligament on each side of the cervix [20]. The nerve supply to the lower vagina derives from perineal branches of the pudendal nerve (S2-S4) [21]. In contrast to the extensive nerve supply of the vulva only a small number of nerve fibers penetrate the epithelium in the vagina. Sensation of light touch and pain are therefore almost absent in the vagina which explains the fact of usually asymptomatic infections and tumour growth in the vagina [19].

Lymphatic system

The whole vagina is surrounded by a fine capillary network of anastomosing lymphatic channels. In spite of these numerous anastomoses the lymph drainage appears to follow a quite regular pattern related to the anatomy of the vagina [19, 22]. The lymphatics of the *anterior wall* drain into the lymph nodes of the lateral pelvic wall or the vesicle lymph nodes and the lymphatics of the *posterior wall* drain into the pelvic, presacral, rectal and aortic lymph nodes. The lymphatics of the *vaginal vault*, as those of the uterine cervix, drain into the pelvic lymph nodes. The lymphatics from the *middle portion* of the vagina usually drain into the superior gluteal lymph nodes. The lymphatics of the *distal vagina* anastomose with those of the vestibule, vulva and anus and drain into the nodes in the inguinal region [22].

EMBRYOLOGY

The vagina develops from the "vaginal plate", a proliferation of epithelial cells from the müllerian tubercle which occur at about the 11th week [19]. The müllerian or sinus tubercle is the region where the müllerian, mesonephric and urogenital sinus epithelia meet. The current opinion is that the vaginal epithelium is of dual origin, the upper three-fifths of the vagina are of müllerian origin, while the lower two-fifths is derived from the urogenital sinus [23, 24]. The vagina is completely canalized by the sixth month, which is quite a late event [19].

NORMAL HISTOLOGY

The vagina is lined by a non-cornified stratified squamous epithelium. The thickness of the epithelium is depending on the function of the ovaries. The mature normal vaginal epithelium can be divided into 6-10 layers of cells where five levels of differentiation can be identified.

There are three layers of the wall of the vagina: a mucosa, a muscular layer and a fibrous layer. The great elasticity of the vaginal wall is due to the large number of elastic fibers in the connective tissue.

Age-related changes in the epithelium and vaginal secretion

The physiological discharge usually has a clear or white colour. The secretion derives from the glands of the cervix uteri and from shed epithelial cells in the vagina. The acidity (pH 4-4.5) caused by the lactic acid protects the vagina against ascending infections. There is a normal cyclical fluctuation with an increase in flow just before ovulation [25]. During the proliferation phase the estrogenic stimulation makes the vaginal mucosa thicker due to a raised cellular maturation. After the ovulation and during the luteal/secretory phase the progesterone stimulation will cause a reduced maturation and increased cellular desquamation.

Postmenopausally, when the cyclic hormonal simulation cease, the epithelium is reduced in thickness and the cellular maturation is diminished. The time to the development of an atrophic epithelium and the degree of atrophy varies considerably [26].

NATURAL HISTORY OF VAGINAL CARCINOMA

It has been supposed that vaginal carcinoma has a similar natural history as cervical carcinoma with the development over a preinvasive phase [27].

Vaginal intraepithelial neoplasia (VAIN) is a rare condition (<1% of all genital intraepithelial neoplasias) [28]. It is usually asymptomatic and diagnosed by cytology. The risk factors are the same as for vaginal carcinoma such as previous cervical dysplasia, prior gynaecological malignancies, prior hysterectomy, HPV and earlier irradiation. VAIN is predominantly located to the upper vagina, is often multifocal (30-40% of the cases) [27, 29] and may occur concomitantly with CIN and VIN. In the literature it has been described that most of the vaginal intraepithelial neoplasia go to remission, while about 5-10% progress to invasive carcinoma in spite of close follow-up [28, 30, 31]. Significant multivariate risk factor for persistance or progression were multifocal lesions [28, 29]. The mean age for VAIN lesions has been reported as 51 years [28, 32]. HPV of different types have been detected in many VAIN lesions and might thus be etiological agents for the preinvasive vaginal disease [33, 34].

HISTOPATHOLOGY

Preinvasive disease

- VAIN 1- mild dysplasia
- VAIN 2- moderate dysplasia
- VAIN 3- severe dysplasia and carcinoma in situ (CIS)
- Postradiation dysplasia

Malignant tumours

The vast majority of vaginal malignancies (80-90%) are squamous cell carcinoma. 4-10% adenocarcinomas, 3% malignant melanomas, 2% sarcomas and 1% other histologies [3, 4, 22, 35].

Invasive epithelial tumours

Squamous cell carcinoma (Figure 1)

According to the terminology proposed by World Health Organization most cancers are of large cell type and keratinizing cancers are less frequent [26]. Squamous cell carcinomas of the vagina are heterogeneous, but usually moderately differentiated. Three gross appearances have been described: nodul-ulcerative, everting or papillary and infiltrating or indurated [36]. The ulcerative pattern is most common [37].

Microinvasive squamous cell carcinoma of the vagina

The criteria for the diagnosis is unclear, but a definition of stromal invasion of less than 2.5 mm in depth has been proposed [26].

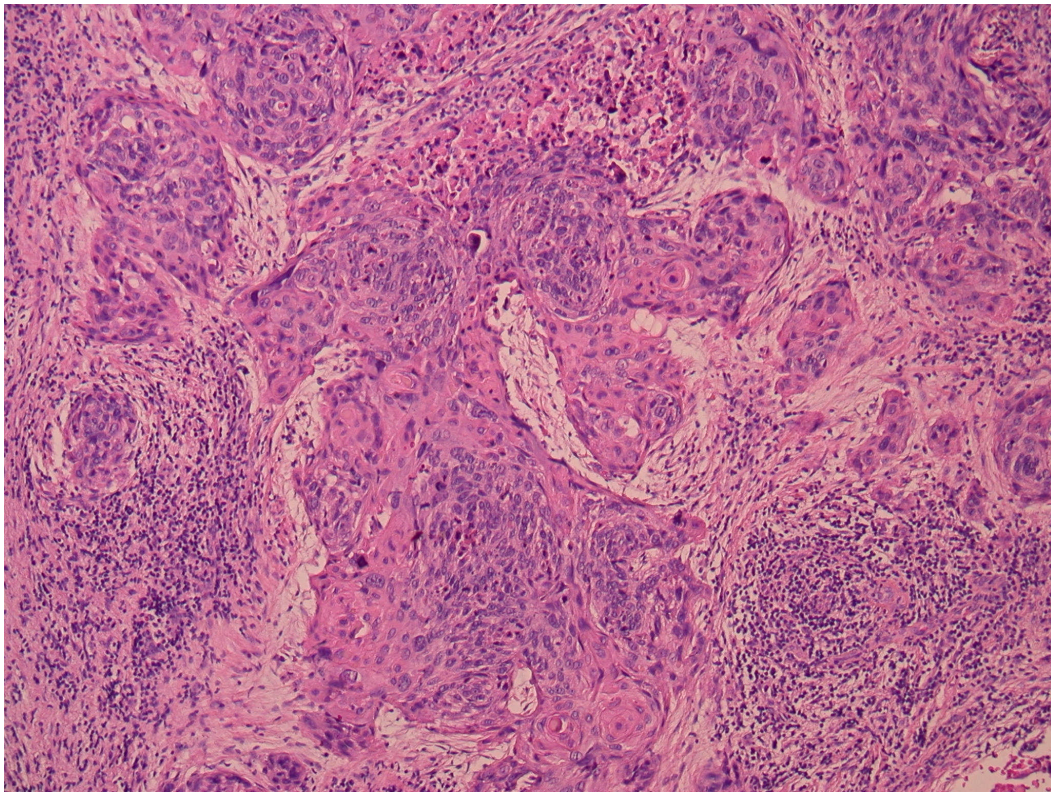Figure 1. Histopathological image of a typical moderately differentiated squamous cell carcinoma of the vagina.

Adenocarcinoma

In the case of adenocarcinoma in the vagina a metastasis from other organs (especially genital and gastrointestinal) must be excluded.

The majority of the adenocarcinomas are of the clear cell (mesonephric) type. They are thought to be preceded by atypical adenosis or glandular dysplasia [38].

Other epithelial carcinoma described in the vagina:
Basal cell carcinoma and adenoid cystic carcinoma [39, 40], synoviod tumour of the vagina [41] and small cell (neuroendocrine) carcinoma which is a rare and aggressive tumour showing similarities with those that appear in the lungs [42, 43].

Non-epithelial tumours
Endodermal sinus tumours, malignant melanoma, sarcoma (leiomyosarcoma and embryonal rhabdomyosarcoma), vascular tumours, lymphomas and stromal sarcomas.

Metastatic tumours
According to a review by Hilborne and Fu 1987 only 16% of invasive vaginal neoplasm represented primary neoplasms, while 84% were secondary [26].
The most common primary site was cervix (32%), followed by endometrium (18%), colon and rectum (9%), ovary (5%), vulva (6%) and urinary bladder and urethra (4%).
Of the squamous cell carcinomas 27% were defined as primary while 73 % were secondary (from cervix and vulva) [26].

CLASSIFICATION AND CLINICAL STAGING

Cases should be classified as vaginal carcinoma when the primary site of growth is limited to the vagina, not involving the cervix, vulva or urethra. Otherwise the tumour should be classified as cervical, vulvar or urethral tumour. The possibility of a secondary tumour from other sites should be excluded. It is sometimes difficult to discriminate between a secondary cervical carcinoma and a primary vaginal carcinoma. As most recurrences from cervical carcinoma occur within 5 years many authors have chosen this limit for a distinction [44], while others have proposed a ten-year disease-free period following pelvic irradiation for cervical carcinoma [45]. However there is no consensus in the literature for these definitions.
The currently used staging classification of vaginal carcinoma was adopted 1963 by the Cancer Committee of the International Federation of Gynaecology and Obstetrics (FIGO) [46], stage IV has later been subdivided to IVa and IVb [4].

Stage 0   Carcinoma in situ; intraepithelial neoplasia Grade III
Stage I   The carcinoma is limited to the vaginal wall
Stage II   The carcinoma has involved subvaginal tissue but has not extended to the pelvic wall
Stage III   The carcinoma has extended to the pelvic wall
Stage IV   The carcinoma has extended beyond the true pelvis or has involved the mucosa of the bladder or rectum (bullous oedema does not permit a case to be stage IV).
    IVa   Tumour invades bladder and/or rectal mucosa and/or direct extension beyond the true pelvis
    IVb   Spread to distant organs

Modifications of the FIGO staging system for vaginal carcinoma have been proposed, including subdivision depending on size of the tumour [37] and of stage II to IIa (subvaginal extension) and IIb (parametrial infiltration not involving the pelvic wall) [45, 47, 48].

Mode of spread

Vaginal carcinoma spreads primarily by local invasion. The thin wall of the vagina, the extensive lymphatic network will make the vaginal tumour grow rapidly and spread early to regional lymph nodes and paravaginal tissues with involvement of the urethra, bladder and rectum. In advanced cases development of fistulas to the neighbouring organs might occur. Tumours in the upper vagina spread to the pelvic nodes while tumours in the lower part spread to both the pelvic and inguinal nodes (for details see above). Haematogenous spread is unusual. The most common sites for distant metastases are the lungs, the liver and the bone.

EPIDEMIOLOGY AND ETIOLOGY

The etiology to vaginal carcinoma is not well known, but has been supposed to be similar as cervical carcinoma. Some case-control studies have been conducted to elucidate epidemiological data and risk factors for vaginal carcinoma, but due to the rarity of PCV, the materials are small and the results are somewhat contradictory [49-53]. The possible etiological factors are summarized in Table 2.

Incidence and geographic differences

There were 1387 cases of carcinoma of the vagina reported to the National Swedish Cancer Registry in the period 1958 - 2003. The age-standardized incidence rate (adjusted to the world standard population) varies between 0.3 - 0.6 per 100000 women but without any certain time-trend [1].
The highest rates of vaginal carcinoma have been found in South America, among black women in the United States and in India [54, 55]. A low rate has been observed among Jewish women [56].

Table 2. PCV. Summary of etiological data.

Etiological factors		
Risk factors	*Other risk factors*	*Possible riskfactors*
Age	Other gynecological dysplasia and neoplasia	Immunosuppression
Earlier CIN, VAIN and/or neoplasia of the cervix	Smoking	Nulliparity
Early hysterectomy	Low socioeconomic status	Estrogens
HPV[1] (and other STD[2])	Earlier pelvic irradiation	High age at menarche
		Low age at menopause
	Unstable marital status	Other malignancies
	Number of sexual partners	
	Early age at first intercourse	
	Vaginal trauma from pessaries, prolapse, high gravidity and/or hygienic factors	

[1]HPV-Human papillomavirus, [2]STD-Sexually transmitted disease

Social characteristics

The age-specific incidence rate is highest after the age of 60, with a peak in the 70-80 year-old age group (Figure 2). Only about 11% are less than 50 years old [55, 57].

Low socio-economic status (low social class, low family income and low educational level) has been found to be more common among cases with vaginal carcinoma than among control cases [49, 50, 56, 58], but in more recent follow-ups the social class differences have diminished [58].

Unstable marital status has been found among patients with vaginal carcinoma with a tendency for an increased risk among separated and divorced women compared to married women [52, 56].

The well established risk factors for cervical cancer (number of sexual partners, early age at first intercourse, smoking and alcohol) have also been observed in the etiology of vaginal cancer [49, 50, 52, 56, 59-61].
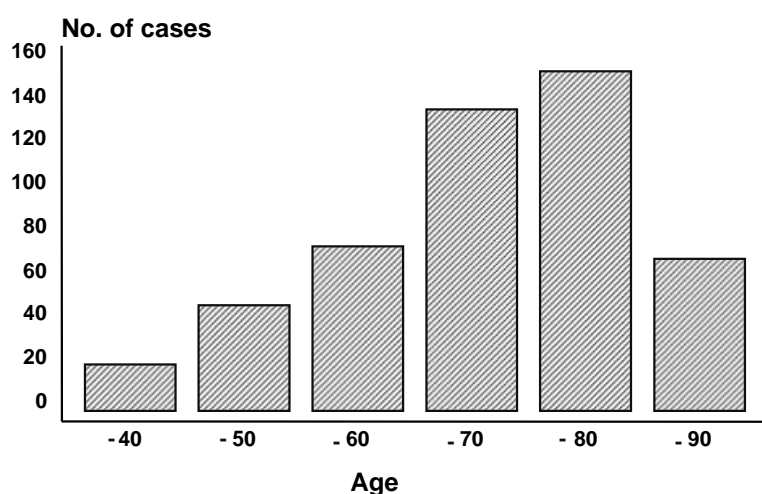
No. of cases

Figure 2. Age distribution for carcinoma of the vagina (1987-93). Pettersson F, ed. Annual report vol 22.

Reproductive data

Menstrual history and parity have been supposed to be involved in the etiology of PCV and therefore frequently investigated in the literature, especially in the older papers. An increased risk for the disease with late menarche (>14 years) and with early natural menopause (<45 years) has been described [49], which compares well the social class variation, that the age at menarche is highest and the age at menopause lowest in lower social classes [58, 62].

Multiparity has been discussed as a risk factor with the hypothesis that high pregnancy rate with injury to the vaginal walls at delivery could be a contributable factor.

On the other hand also nulliparity has been considered a risk factor with the hypothesis that the reason for nulliparity may be a pelvic inflammatory disease, caused by a sexually transmitted agent, which might also be the risk factor for vaginal carcinoma.

However, in the case-control studies by Brinton et al and Daling et al no correlation between parity and PCV was observed [49, 50].

Other diseases

The metabolic syndrome (obesity, diabetes mellitus and hypertonia) has been pointed out as a possible risk factor for PCV [64], while other studies have not verified any association [36, 49].

Young women with immunosuppressive disorders such as HIV and concomitant HPV infection have been described to be at increased risk for rapidly progressive vaginal carcinoma [65].

Human papillomavirus (HPV) has been described in the literature to be a risk factor for developing vaginal carcinoma [50, 51, 53, 66-68]. HPV has been detected in 21-76% (average 53%) of vaginal carcinomas with HPV-16 as the most common subtype [50, 51, 53, 67, 68]. Other infection agents found to be associated with the disease are HSV-2 [50, 51, 53] and Chlamydia trachomatis [53]. However, the strongest effect was observed when antibodies against those two agents occurred simultaneously with antibodies against HPV-16 [53]. Other sexually transmitted diseases such as syphilis, gonorrhea and granuloma inguinale have been reported to occur prior or concurrent with vaginal carcinoma but the significance of this is unknown [56, 69-71].

Cancers of other sites prior to the vaginal carcinoma

The theory of multicentric primary gynaecologic neoplasms have been emphasized by several authors [55, 72-74] and a statistically significant increased risk for secondary gynaecologic neoplasms in women with cervical, vulvar and vaginal carcinoma has been found [73]. Vaginal cancer preferentially follows cervical cancer. According to earlier studies 30% of patients with PCV had prior CIS and/or invasive cancer [50, 72, 75].

An association between other primary carcinomas and PCV has been shown, especially carcinomas related to smoking [59, 76, 77], but also carcinomas related to HPV infection (other than cervical- and vulvar carcinomas) such as carcinomas of the upper aerodigestive tract, nonmelanomatous skin cancers and anal carcinomas [59, 77-79].

Little is known about the importance of genetic factors for the development of vaginal tumours. Rose et al found that 25% of the patients with multiple gynaecologic cancers had a family history of cancer, but the degree of kinship was incomplete in that study [73].

Treatments/surgery prior to the vaginal carcinoma

Hysterectomy

An excess risk for developing PCV associated with previous hysterectomy irrespective of the reason was found in some studies [49, 70, 80, 81]. The risk was highest in women who reported a hysterectomy prior to age 40 [49]. Daling et al 02 also found an increased risk for PCV but only among hysterectomised patients with no prior anogenital cancer [50]. However, in another study no increased risk for PCV was observed in patients hysterectomised for benign disease [82]. Prior hysterectomy has been reported in 37% (range 9-59%) of patients in earlier studies [27, 44, 49, 64, 70, 80, 83-89]. In patients with prior hysterectomy due to dysplasia or neoplasia of the cervix, PCV could be caused by the same etiological agent and/or residual disease in the upper vagina due to unclear surgical margins. However with regard to the

multicentric nature of the disease clear surgical margins might not assure a total eradication of the disease [27, 75].

Pelvic radiation therapy

Pelvic radiation therapy, especially for prior cervical disease, has been pointed out as a risk factor for developing carcinoma of the vagina [45, 75, 90-92]. Women who were under 40 years when irradiated for cervical carcinoma will be at high risk of developing PCV 10 to 40 years later [72, 93]. However, data from another study suggest that radiation induced vaginal cancer is quite a rare event [73].

Chronic irritation and trauma

Chronic irritation and trauma to the vagina caused by uterovaginal prolapse, long-term pessary use, vaginal plastic surgery, hygiene factors (vaginal douching) and chronic vaginal discharge have been associated with vaginal carcinoma [49, 56, 84, 90, 94-96]. The mechanism might be that the continuous trauma to the vaginal epithelium from the prolapse and/ or pessary induce tissue repair processes and may result in an ulceration and malignant transformation of the epithelial cells leading to the development of VAIN and invasive squamous cell carcinoma [96, 97].

Hormones

Some cases of vaginal carcinoma have been found among patients treated with corticosteroids. In vitro studies have shown that steroids (dexamethasone) increased the expression of HPV E6 and E7 [98, 99], which in addition to the immunosuppressive condition caused by the steroid therapy might contribute to the development of PCV.
There is clinical evidence that early VAIN (vaginal intraepithelial neoplasia) lesions in postmenopausal women should be treated with estrogens applicated vaginally as a firstline treatment. This may mature the vaginal epithelium and thus converting the surface epithelium to normal [100]. Otherwise there is not much data in the literature concerning the use of exogenous estrogens and vaginal carcinoma. Brinton et al did not find any elevated risk neither with the use of oral contraceptives nor menopausal estrogens [49]. Studies on cervical carcinoma have revealed increased risk with the duration of steroid usage [98]. It has been demonstrated that steroid hormones (both estrogen and progesterone) enhance the transcription of HPV E6/E7 proteins and might thus be essential cofactors in the HPV(16)-mediated cervico-vaginal carcinogenesis [98, 101-103].
Clear cell adenocarcinoma related to intrauterine exposure to diethylstilbestrol (DES), a nonsteroidal estrogen, used in the United States from 1940 to 1971 to prevent miscarriage, has been discussed frequently [104]. They occur in young women with a peak incidence at 19 years [105], but are diagnosed less frequently in recent years as prescription of DES has been discontinued. In Sweden DES has not been used and no cases have been reported in our studies.

CLINICAL FEATURES

The most common presenting symptoms are vaginal bleeding and discharge (60-85%) [37, 70, 75, 106]. Other less common symptoms are pelvic pain (10-20%), presence of a mass or vaginal prolapse (10%). Pelvic pain is usually a late symptom related to the tumour size and the reaction from the surrounding tissue. Other symptoms often related to the position of the vaginal tumour are bladder symptoms (20%) from anterior tumours and bowel symptoms from posterior tumours [75, 107]. Some patients are asymptomatic (10-25%) preferably those with early stage disease and the diagnosis is then made by routine gynaecological examination or by smear taken in the screening programme for cervical carcinoma [70, 75, 106, 108].

Viiki et al provided evidence that positive cytology, when taking Pap-smear in the screening program, followed by negative histology significantly increased the risk of vaginal carcinoma which was greatest during the first year of follow-up and persisted for more than five years [108]. Brinton et al also found that a previous abnormal Pap-smear significantly elevated the risk for vaginal carcinoma [49]. Furthermore other studies have shown that 80% of VAIN lesions are detected in women with prior, concurrent or subsequent CIN and VIN [27, 100].

TREATMENT

Radiation therapy

Radiation therapy (brachytherapy (BT) and/or external radiation therapy (ERT)) has been regarded as the standard treatment for invasive vaginal carcinoma in all stages [3, 83, 88, 106, 107, 109-112]. According to the Annual report [4] 82% of the patients received radiotherapy, either alone (62%) or in combination with surgery (20%). In 15% of the cases, primarily with stage I disease, the treatment was surgery alone [4]. Brachytherapy has been recommended for superficial early stage vaginal carcinoma [113], whereas for more advanced stages combined treatment modality (ERT+BT) results in superior local control and survival [88, 109, 113, 114]. The external radiation therapy is often delivered with a four field technique, 40-70 Gy, 1.8-2.0 Gy daily, 5 days a week over a period of 5-6 weeks. The target volume usually includes the entire pelvis with the primary tumour and the pelvic lymph nodes.

The reported results are similar from high-dose-rate (HDR, >12Gy/h) and low-dose-rate (LDR, <2Gy/h) brachytherapy [113]. The use of interstitial brachytherapy instead of intracavitary treatment has been advocated by some authors in order to obtain better dose distribution and thus improved local control [88, 111, 113, 115-117].

The radiation therapy ought to be individualised and tailored according to the size, site and extent of the vaginal tumour and also with regard to the age and the general condition of the patient [114]. The complex lymphatic drainage of the vagina should be considered when planning the therapy. The most frequently affected lymph nodes are the external, internal and common iliac nodes [22].

However according to the majority of earlier studies the primary failure site for vaginal carcinoma is local recurrence (vagina and paracolpol tissues), with rare initial failures in the external, common iliac and presacral nodes [37, 83, 112]. Against this background Yeh et al found that without decreased control rates, it was possible to use smaller pelvic fields where the upper border of the field should not extend beyond the lower limit of the sacroiliac joints except for advanced stages [112].

Chemotherapy

The knowledge concerning chemotherapy in PCV is limited with regard to the rarity of the disease. Chemotherapy has mostly been used as palliative treatment. However recently chemotherapy given concurrently with radiation therapy (chemoradiation) has been advocated for yielding superior survival in advanced stages (II-IV) of PCV [114, 118, 119] in agreement with the experiences of cervical carcinoma [120, 121]. The chemotherapy used in the combined treatment modality for vaginal carcinoma has been cisplatinum, 5-fluorouracil (5-Fu) or mitomycin-C [114, 119]. Neoadjuvant chemotherapy with cisplatinum and epirubicin to patients with advanced or recurrent squamous cell and adenocarcinoma of the vagina has been described [122, 123], but the results have been desolated.

Surgery

Surgery may be an appropriate therapy for patients with early stage disease of PCV [118]. Surgery followed by selective radiotherapy has been found to improve survival in early stages [94, 95, 124]. Small lesions in the upper vagina may be treated by upper vaginectomy and radical hysterectomy with pelvic lymphadenectomy [95, 118]. Lesions in the distal vagina can be treated by partial vaginectomy, vulvectomy and inguinal lymph node dissection [95, 118]. Advanced stages of PCV with bladder or rectal involvement but not fixed to the pelvic sidewalls requires exenteration (anterior, posterior or total) with the enclosed problems of stomata [95, 118]. Surgery results in altered anatomy and loss of vaginal function with morbidity depending on the extent of the surgical procedure. Radiotherapy has therefore become the most common treatment for PCV as both the anatomy and the vaginal function are better preserved. For patients with recurrent disease after radiotherapy, surgery may be the treatment of choice.

Complications of radiotherapy

Vaginal stenosis of different degrees is the most common complication after radiation treatment for PCV with an occurrence of 25-40% [45, 70, 106, 116]. This complication is most likely associated with the intracavitary treatment but also with the tumour size and stage of the disease [27]. Fistulas, vaginal necrosis, bowel and bladder complications have been reported to occur in 10-20 % of the cases [45, 65, 70, 83, 87, 89, 106, 109, 111]. Major complications are correlated with stage and type of treatment [83, 109], the irradiated volume [112] and the total dose delivered to the tumour [65]. Most complications appear within five years, but some may occur beyond this time, up to 16 years after treatment [83]. Fistulas and bowel complications are especially frequent when previously irradiated patients are retreated with radiotherapy no matter how long the interim period may be [72].

SURVIVAL

The overall 5-year survival rates have improved during a 30-year period (1959-89) with 15% [4] (Table 1). This is most likely due to advances in the irradiation technique and increased knowledge in radiobiology, but also due to earlier diagnosis of the vaginal carcinoma. However, the prognosis is still relatively poor with an overall 5-year survival rate of 47% [4] (Figure 3).
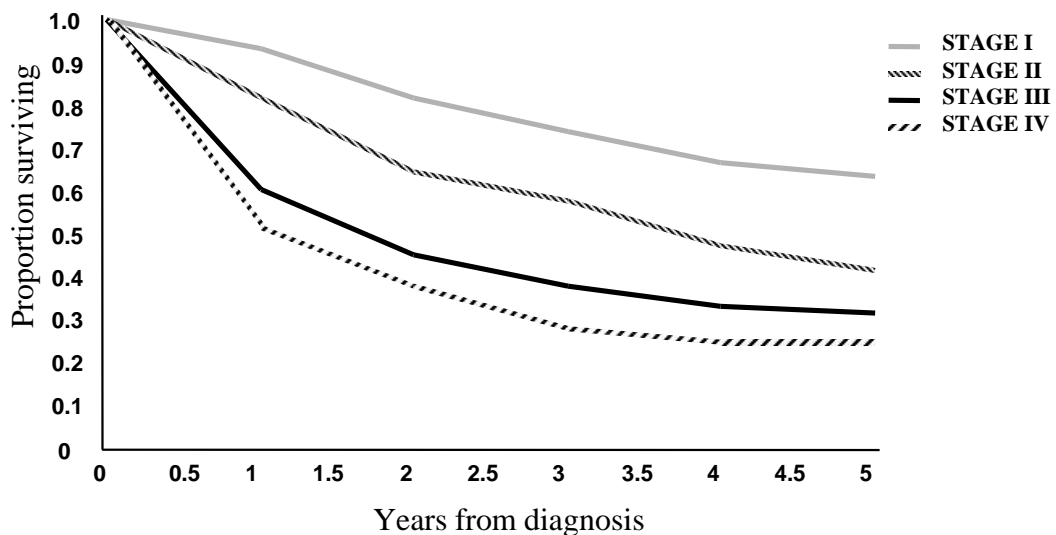

Figure 3. Carcinoma of the vagina. Actuarial survival by stage. Cumulative proportion surviving. Pettersson F, ed. Annual report vol 22, 1994.

PROGNOSTIC FACTORS

According to the literature clinical stage is the most important factor predicting survival. An analysis of 916 cases obtained from the National Cancer Institute's Surveillance, Epidemiology, and End Results program of vaginal cancer, indicated that FIGO stage, histologic grade, lymph node status and patient´s age at diagnosis were independent prognostic factors. Additional analysis of the data by combinations of the independent prognostic factors indicates that the interaction of factors may be more predictive of outcome than any separate factor [126].

Another important prognostic factor, not studied by Kosary et al, is the tumour size, where tumours <5cm are considered to have best prognosis [83, 106, 114]. In paper II in this thesis, which comprising one of the largest material of PCV, the independent prognostic factors observed were stage, tumour size and age at diagnosis.

Studies on the prognostic significance of histology, histologic grade and tumour localisation have produced contradictory results. According to some studies adenocarcinoma in the vagina has worse prognosis than squamous cell carcinoma [83, 116], while others have not detected any difference in survival [106, 127]. Poorly differentiated tumours have been associated with worse prognosis in some studies [106, 107, 109], but not in others [128, 129]. Tumours located in the upper vagina have been ascribed better survival than tumours in the lower part [107]. However this finding has not been confirmed by other authors [109, 116].

Concerning the prognostic impact of biological factors in PCV little is known to date.

CARCINOGENESIS

In the beginning of 1800 cancer was described as a cancerous infection with an "irresistible tendency to invade and destroy structures and with a power of self-dissemination" [130]. In the 1840s a German microscopist, Johannes Mueller, showed that cancer was made up of cells [130, 131]. It had further been noticed that cancer not infrequently appears in several members of the same family [130]. In 1890 David von Hansemann observed mitotic disturbances in cancer cells which divided into three or four cells, instead of two as normal cells, and further he discovered that the chromosomes were changed in shape and number [132]. In 1914 Boveri declared the somatic mutation theory of cancer [133, 134]. In the 1920s it was noticed that there could be a long latent period (up to 30 years) between the first exposure to a carcinogenic chemical and the development of cancer, referred to as the precancerous stage [135, 136]. The two events were termed initiation and promotion [137]. Evidence for the two-stage mechanism in carcinogenesis was obtained by the studies of Berenblum and Shubik [138]. In the 1950s Armitage and Doll presented the hypothesis that cancer is developed through a complex multistage process [139], which has become generally accepted. It is believed that at least four to six genetic alterations are required that occur stepwise [140, 141]. The first genetic damage (initiation) is either inherited or acquired.

During the last 30 years there have been a rapid increase in the knowledge of tumour biology due to the advancements in biological technology, that have made it possible to study genetic changes in the cancer cells and the affected proteins and thereby identifying diagnostic, prognostic and therapeutic markers.

The mechanism of growth control in normal cells, benign tumours, in situ tumours and malignant tumours has been widely studied but is yet not clearly understood.

Normally the stimulatory and inhibitory factors of growth control are strictly in balance until growth stimulus is required either for repair or due to extra work from a specific organ.

In malignant tumours all cells appear to share similar changes in cell physiology, termed "acquired attributes" including ability to generate their own stimulatory and mitogenic signals, resist growth-inhibitory signals, avoid apoptosis, proliferate without limits, undergo angiogenesis and become invasive and metastasize [141].

The cancer genes frequently affected are proto-oncogens that are activated (ex K-ras, c-myc, bcl-2, EGFR) and tumour suppressor genes that are inactivated (ex TP53, RB1, PTEN) and the DNA repair genes that are defect (ex mis match repair genes).

The proteins of these genes act through complex networks of different cellular pathways that are closely connected. Recent research on experimental malignant cell transformation indicate though that disruption of a limited number of pathways will be sufficient for deregulation of the cell cycle and tumourigenesis [142].

Cell division cycle and its regulation

During the last decade the knowledge has increased immensely concerning the regulation of the cell cycle, in which the cell duplicates its genetic material and divides into two identical daughter cells. The cell cycle can be broken down into four different phases: the S (synthesis) phase and the M (mitosis) phase, which are separated by two gaps known as G1 and G2. During G1, the cells "decide" in response to a variety of extracellular and intracellular signals as to whether to proceed into S phase or be

16

withdrawn from the cell cycle and become quiescent or terminally differentiated (G0). The final decision is made near the end of the G1, termed the "restriction point". Once the cells have entered S phase, they are committed to complete the cell cycle and divide and does not respond to external signals. Most of the cells in the body of adults are in G0. Only cells in G0 and G1 can respond to extracellular proliferative stimulation. The transition between the phases in the cell cycle is determined by different classes of cellular proteins [143] (Figure 4).
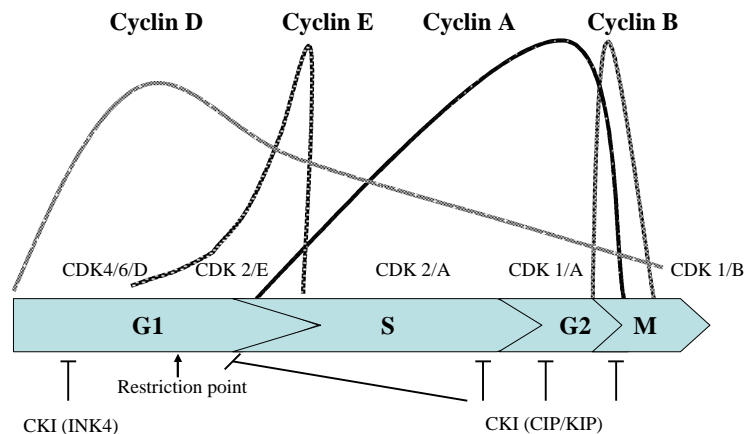

Figure 4. Cycline dependent kinases (cdks) drive the cell cycle forward, but only in association with a regulatory subunit, called cyclin. The cyclins appear and disappear in a carefully controlled "cyclic" pattern mediated by transcriptional activation of the cyclin genes and degradation of the proteins. The activity of the different cdks is also regulated by binding of cdk-inhibitors (CKI). The cyclins and the cdks can be grouped according to their activity in the different phases:
In **G0** and early G1 the cdks are suppressed by high levels of CKI and low levels of cyclins. **G1**: cdk4 and 6/cyclin D1-3, cdk2/cyclin E, in **S**: cdk2/cyclin A and E, **G2**: cdk1and 2/cyclin A and cdk 1/cyclin B and in **M**phase: cdk1/cyclin A and cdk1/cyclin B
The CKI constitute two families: the INK4 family (p15^{INK4b}, p16^{INK4a}, p18^{INK4c}, p19^{INK4d}) and the CIP/KIP family (p21^{CIP1}, p27^{KIP1}, p57^{KIP2}).

To control the progression of the cell cycle there are a number of regulatory pathways, for instance pRb and p53 pathways, acting through checkpoints. Crucial checkpoints are in G1/S (the restriction point) (Figure 4), G2/M and in M phase (the spindle checkpoint). In the absence of mitogenic signals or in case of DNA damage/error the checkpoints are activated, preventing the cell cycle from progression.
The CKIs of the INK4 family act only at the G1 checkpoint, while the CIP/KIP family acts in all the cell cycle phases (Figure 4).
The protein product (pRB) of the retinoblastoma tumour-suppressor gene (RB-I, chromosome 13q14) acts as a transcription factor and plays an important role in regulating the ability of cells to enter S phase [144]. In G1, when CDKs are absent the hypophosphorylated retinoblastoma protein (pRb) remains associated with E2F.
In the presence of mitogenic signals Cyclin D starts to accumulate forming CDK4/6-Cyclin D complex which phosphorylates pRb and releases E2F [145]. In the activated form E2F stimulates transcription of genes encoding proteins necessary for S-phase entry, for example Cyclin E. The CDK2/Cyclin E holoenzyme formed in the late G1, is

important for passing through the G1/S boundary. For fully active CDK2 p27^{KIP1} has to be degraded. p27^{KIP1} is consequently considered to be a key mediator of the G1/S transition.

p53 is a 53 kDa nuclear phosphoprotein coded by the p53 tumour suppressor gene located on chromosome 17p13. It acts primarily as a transcription factor and is regarded as the guardian of the genome [146]. In normal cells the levels of the short life p53 ("wild type") are low, suppressed by its negative regulator MDM2. In response to cellular stress (DNA damage from drugs and ionizing radiation, hypoxia, oncogene activation) phosphorylation of p53 by protein kinases, such as ATM and p19^{ARF}, blocks MDM2 binding resulting in a cascade of modifications that activate p53 DNA binding on target genes such as p21^{CIP} , Bax and GADD45 leading to cell cycle arrest, apoptosis or DNA repair [147]. p21 is an essential downstream mediator, acting on a variety of cyclin/CDK complexes, thereby p53 initiated block can occur in both G1 and G2/M phases [148, 149]. The p21 gene is located on chromosome 6p21 [150]. The GADD45 gene product interacts with different cell cycle proteins such as PCNA and contributes to DNA repair and G2/M cell cycle arrest [147].

Cell cycle disturbances in cancer

Cancer is commonly considered a disease of the cell cycle, since it originates from the functional suppression of negative regulators (often encoded by tumour suppressor genes- p53 pathway and the Rb pathway) and abnormal expression or activation of positive regulators (often encoded by proto-oncogens) [143, 151].

Inactivation of negative regulators

The p53 and pRb pathways have been widely studied the last two decades and many, if not all, human tumours contain mutations that directly or indirectly interfere with the function of p53 and pRB [144, 152]. Inactivation of p53 function, either by mutation of wild type p53 or mutation-independent mechanisms, hamper cellular processes such as cell cycle arrest and apoptosis, with a concomitant loss of cell cycle control [153].

Mutated p53, which has a longer half-life, than the wild type, accumulates in the cell and is detectable with immunohistochemical methods. According to recent reports it is evident that p53 gene mutations correlate with the clinical aggressiveness of a tumour [154, 155]. However studies on different gynaecological malignancies have failed to show that p53 is an independent prognostic variable [156].

HPV oncoprotein E6 and E7

In cervical cancer mutations of the p53 and pRb genes are rarely detected. The principle causative agents for cervical carcinoma, the human papillomavirus (HPV) serotypes 16 and 18, express the oncoproteins E6 and E7 which interact with p53 and pRb respectively, leading to inactivation of these proteins. By binding to pRB E7causes disruption of the pRb/E2F complex and thereby activating the cell cycle by overcoming the G1/S checkpoint control in DNA-damaged cells [157]. E6 complexes with p53 leading to degradation of the protein. Other HPV (6 and 11) are considered as low risk types mainly due to a lower ability of the oncoproteins E6 and E7 to interact with tumour suppressors and inactivate them and other regulatory proteins [158].

HPV is detected in 50-80% of all cases of vaginal carcinoma [50, 159]. Overexpression of p53 have been found in 20-50% of PCV [159, 160], while no alterations in the pRb expression have been shown [161]. Thus inactivation of p53 might occur either by

mutation or mutation-independent mechanisms (HPV E6) in vaginal carcinogenesis. Likewise in vulvar carcinoma HPV is found only in a small fraction of the cases [162, 163], and p53 overexpression has been demonstrated in 40-80% [162, 164].

Increased levels of p21 have unexpectedly been seen in many cervical carcinomas, which thus might occur via p53 independent pathways, for example by E2F which is generally activated in HPV infected cells. However the oncoprotein E7 can associate with p21 and also with p27, inactivating their functions, without degrading them.

The prognostic importance of p21, p27 and p16 has been investigated in numerous studies with conflicting results [165]. In a recent study concerning cervical carcinoma and expression of p21, p27 and p16 no independent prognostic association could be found [166].

Induction of positive regulators

High cyclin A expression has been reported in vulvar and cervical carcinoma [167, 168]. In cervical carcinoma this was correlated with poor prognosis [168], but not in vulvar carcinoma [167]. In **paper IV** a high expression of cyclin A was found in the majority of PCV. Overexpression of cyclin E has been found in cervical carcinoma [169, 170], but there was no correlation with the prognosis [169]. On the other hand, in breast cancer cyclin E has been reported to be the strongest independent predictor [171]. Cyclin E overexpression in cervical carcinoma might be the direct result of the HPV E6 and E7 proteins. HPV E7 leads to an inactivation of pRb and thereby to an increased transcription of cyclin E (by E2F). In cervical carcinomas, that express HPV E6/E7 oncoproteins, the most characteristic features appear in the up-regulation of cyclin E and p16 expression, which also might be used for diagnostic purposes in order to discriminate between benign, dysplastic and neoplastic lesions [157, 172-174].

The most commonly studied oncogenes in gynaecological cancers are c-myc, k-ras and growth factors such as c-erbB2 and EGFR (epidermal growth factor receptor) [156]. Overexpression occurs in various degrees and there are conflicting data concerning the prognostic impact of overexpression of these genes in ovarian, endometrial and cervical carcinoma [156]. In vaginal carcinomas overexpression of c-erB2 and EGFR have been detected only in a minority of the cases, and no correlation with prognosis was shown [161]. In cervical carcinomas overexpression of c-erbB2 was detected in 40% of the cases [175-177], but was found to be uncommon in another study [178].

The product of the c-erbB2 (or HER-2/neu) oncogene is a growth factor receptor with tyrosine kinase activity. The receptor tyrosine kinase (RTK) (one type of GFR), has been implicated in the development of human neoplastic diseases. The RTKs are activated by ligands (ex EGF, PDGF) binding to their extracellular domain (ex EGFR, PDGFR) and activates the protein tyrosine kinase resulting in a cascade of intracellular signals, which affects various genes, for example Ras oncogene [179].

Therapies targeting tyrosin kinases have been developed: Herceptin, Gleevec and Iressa [180].

Cell proliferation markers

Different techniques have been developed to asses the proliferation (growth fraction) of a tumour [181]. An early method is to determine the S-phase fraction by means of image- or flow-DNA content cytometry [182]. Other methods are measuring of

immunohistochemical expression of nuclear proteins such as Ki-67, PCNA (proliferating cell nuclear antigen) and cyclin A.

The general proliferation marker Ki-67 is expressed in all phases of the cell cycle except G0 [183]. In a variety of tumours (for instance breast and prostate carcinoma), the Ki-67 expression has repeatedly been proven to be of prognostic value for survival and tumour recurrence, while no such correlation has been demonstrated for other tumours (for instance cervical carcinoma) [184, 185]. However, the functional significance of the protein still remains unclear [185]. The disadvantage of the first monoclonal antibody against Ki-67, described in 1983 [186], was that it could only work on fresh tissue specimens. This was overcome in 1992 when the monoclonal antibody MIB-1 was available and could be used in formalin-fixed paraffin sections [187].

PCNA interactes with several cell cycle regulators and is essential for DNA replication and repair. It is synthesized in late G1, S and also in G2/M phases of the cell cycle. It therefore cannot be used as a reliable S-phase marker.

Cyclin A starts to accumulate during S phase and is believed to be important for initiation of DNA replication. In mitosis phase before metaphase it is abruptly destroyed. The cyclin A gene is located on chromosome 4q26-q27 [188] and synthesis is controlled by E2F and other transcription factors [189].

INVASION AND METASTASIS

The molecular pathways that lead to invasion and metastases still remain unclear.

Degradation of extracellular matrix (ECM) macromolecules in the basement membrane, such as collagens, laminins and proteoglycans is considered one of the first steps in tumour invasion and the development of metastases. It has been found that many malignant tumours have higher levels of proteases and collagenases than the corresponding normal tissue why it has been generally accepted that tumours produce and secrete lytic enzymes that degrade the normal tissue [190]. Triggered production of matrix metalloproteinases (MMP-2) in metastatic cells, but not in non-metastatic cells has been demonstrated [191].

Angiogenesis is a major event in tumour growth and metastasis as the tumour requires blood supply to receive nutrients and oxygen for survival. A potent inducer of angiogenesis was shown to be VEGF, a protein released from the cancer cells acting at specifically receptors on endothelial cells [192]. Mutations of Ras and/or p53 are thought to be causative for tumour-associated angiogenesis by regulate the expression of VEGF [193].

Laminin-5 and tumour invasion

Tumour invasion is characterized by the tumour cells crossing the basement membrane (BM) marking the difference between a malignant and benign tumour [194]. Laminins are molecules of the basement membranes (BM), which are thin, dense sheets of ECM, separating the epithelium from the underlying connective tissue. The laminins constitute together with type IV collagen, nidogen and perlecan the major components of the BM. They are thought to have an important role in cell adhesion, migration, differentiation, proliferation, survival, angiogenesis (Ln-8, 10) and metastasis [195].

The laminin family is a group of large cross-shaped heterotrimeric proteins composed of three subunits: one heavy α- chain and two light β- and γ-chains [196, 197]. The first

laminin (laminin-1, α1β1γ1) was isolated more than 20 years ago and in the end of the 1980s several homologs of these chains were isolated. At present at least 12 different laminins are identified [195]. They are synthesized by numerous cell types and expression of laminin isoforms are cell- and tissue specific [195] with different biological functions [197].

Laminin-5 (Ln-5) (also referred to as kalinin, nicein, epiligrin or ladsin) is produced only by the epithelial cells [198] and consists of, α3, β3 and γ2 chains [196, 199] (Figure 5). These three subunits are coded by specific genes the LAMA3 gene on chromosome 18q11.2 (α3,), LAMB3 on chromosome 1q32 (β3) and LAMC2 on 1q25-31 (γ2) [200, 201]. Laminin-5 plays an important role in epithelial cell adhesion to the basement membrane forming hemidesmosomes by binding integrin receptors. Integrins recognize mainly laminin α chains, but may also recognize β and γ chains, hoewever these interactions are less well understood [195]. The C-terminal domain binds to cell surface receptors, providing cell anchorage and thereby signal different regulating pathways and expression of specific genes. With their N-terminal parts, laminins interact with extracellular matrix proteins mediating cell-ECM interactions [202].
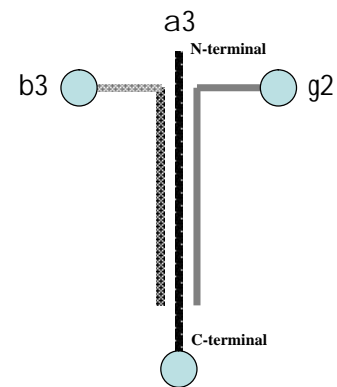

Figure 5. The heterotrimeric laminin-5 molecule.

Studies have shown that the γ2-chain of Ln-5 is expressed by the migrating keratinocytes but not by the stationary ones in wound healing in skin [200, 203]. In epithelial tumours there is an intracellular (cytoplasmatic) accumulation of the γ2-chain located at the epithelial-stromal junction, the invasion front [204]. In invading cells, overexpressed laminin γ2 chain appears to be present in a monomeric form, as laminin α3 and β3 chains are absent or found at much lower levels [195].

Ln-5 can act as a motility factor and at other times as an adhesive factor, depending on the state of proteolytic processing [194, 195]. Plasmin cleavage of laminin α3 chain makes Ln-5 adhesive [205], whereas cell-surface metalloprotease MT1-MMP and, to a lower extent, MMP-2 triggers migration by cleavage of the N-terminal regions of the γ2-chain [195, 206, 207].

Several immunohistochemical studies have reported that Ln-5γ2-chain expression is up-regulated in invading cancer cells, suggesting it as a marker of invasiveness in squamous cell carcinomas and other epithelial malignancies [199, 204, 208-213]. Increased expression of the γ2-chain has also been correlated with a more aggressive behaviour of the tumour [209, 214, 215], which has also been observed in PCV (**paper III**). However, according to some reports the expression of the Ln-5γ2-chain seems to be reduced in the course of malignancies, which might be indicative of metastatic potential [194, 211]. In studies of breast and prostate cancer a down-regulation of Ln-5γ2-chain has been reported [198, 216, 217]. Furthermore the expression of the γ2-chain has been revealed to be a marker of microinvasiveness in cervical and vulvar carcinoma [218, 219]. Immunohistochemical detection of Ln-5γ2-chain is the most common method, using both mono- and polyclonal antibodies [220]. The N-terminal part of the γ2-chain released from the invading tumours can be immunodetected in the biological fluids with a potential use in clinical diagnosis of invasive tumours [221].

GENOMICS

Over the past decade, our knowledge of genomics (the study of the human genome) in malignancies has increased enormously. In 1956 the correct number of chromosomes in man was finally established (44 autosomes and 2 sex chromosomes) by Tjio and Levan [222]. Hitherto, in the Human Genome Project [223] 20-25000 genes have been identified (approximately 98% of the human genome) and the National Cancer Institute (NCI) has established the Human Tumour Gene Index, which is available over the Internet [224].

Nuclear DNA cytometry

Methods for the assessment of nuclear DNA content have gradually developed since Caspersson 1936 introduced microspectrophotometry [182, 225].
Two cytometric methods are generally used, flow cytometry (FCM) and image cytometry (ICM).
Both methods can be performed on fresh, frozen or formalin-fixed paraffin-embedded tissue. In flow cytometry, the most common method, cell nuclei are stained with a fluorochrome. The nuclear fluorescence in the cell suspension is quantified by the flow cytometer. The amount of fluorescence is assumed to be equivalent with the amount of the DNA [182]. An advantage of this method is the capability to rapidly analyze large cell populations (approximately 20000 nuclei per specimen). A disadvantage is however the inability to distinguish between normal and tumour cells. Further the cell dispersion must be entirely monocellular otherwise there might be errors.
Image cytometry is based on Feulgen staining, a method described by Feulgen and Rossenbeck 1924 [182, 226]. With the image method the DNA staining can be measured on microscopic slides and 100-500 whole cells are microscopically selected for the analysis. Thereby small tumour populations within large stromal components can be successfully analysed with ICM, in contrast to FCM where small subpopulations of aneuploid tumour cells may be overlooked [227, 228].
Nuclear DNA content can be of value both as a diagnostic and a prognostic marker. The ploidy pattern generally remains unchanged throughout tumour progression. Distinct aneuploidy can be observed early in the carcinogenesis making it possible to differentiate between different cellular alterations (non-specific, premalignant and malignant) [155].
Numerous studies have confirmed DNA ploidy as a prognostic factor for endometrial, breast and prostate carcinoma, dividing the tumours into two types: diploid, that progress slowly and aneuploid, that progress rapidly [155, 229, 230]. Cervical carcinoma most often show DNA aneuploidy and studies have failed to verify any prognostic significance depending on DNA content [156, 231]. In vulvar carcinomas there are contradicting results with regard to the prognostic impact of the nuclear DNA content [156]. Most vaginal carcinomas have been observed to be aneuploid (**paper IV**).

Comparative genomic hybridization

CGH is based on quantitative two colour fluorescence in situ hybridization first described by Kalloniemi 1992 [232]. It produces a map of DNA copy number changes throughout the entire genome, visualized as gains and losses on a karyogram.

The major advantage of CGH is that it allows a screening of genetic changes in the entire genome. The limitation is that CGH only detects changes being present in a substantial proportion of tumour cells. It does not reveal translocations, inversions, and other alterations that do not change copy number. By contrast, unbalanced genomic aberrations predominate in solid tumours, especially in carcinomas, making these tumours ideal targets for CGH analysis [232, 233].

Increase of DNA copy number changes, especially high-level amplifications is clearly associated with tumour progression and a marker of increased genomic instability. Benign tumours may have copy number changes, but not numerous amplifications, a finding that might be helpful in the differential diagnosis between malignant and benign lesions [234]. Studies have shown that DNA ploidy changes and impairment of p53 function precede the appearance of recurring chromosomal copy number changes in the majority of cells [235, 236].

The average number of copy alterations (ANCA index) is a measure of the number of acquired chromosomal copy alterations in a given tumour type. The ANCA index increases with increasing cellular atypia and aggressiveness, which has been shown in cervical, colon and breast carcinogenesis [235]. For example dysplasia of cervix (CIN II-III), stage I and stage IIb-IV have ANCA values of 0.4, 4.0 and 8.2 respectively [237, 238]. Primary carcinoma of the fallopian tube has an extremely high ANCA value of 19.7, indicating a highly aggressive tumour [239].

With CGH specific chromosomal aberrations can be mapped during genesis of solid tumours. For example in colorectal carcinomas copy number increases on chromosome 7 and 20 are early events in the colorectal carcinogenesis, occurring in high-grade adenomas. In cervical carcinoma a gain of chromosome arm 3q is found in 90% of stage I carcinomas, while rarely detected in severe dysplasias. In more advanced stages additional alterations are identified, such as high level of copy number changes of chromosome arm 5p [235]. Likewise in squamous cell carcinomas of the vulva, as well as in vaginal carcinoma (**paper IV**), gain of chromosome arm 3q seems to be an early but consistent event during genesis [240, 241].

The region of minimal overlap points to chromosome band 3q26 contain the gene for the RNA component of human telomerase (TERC). Detection of 3q gain and amplification of TERC in routinely collected Pap smears may assist in identifying low-grade lesions with a high risk of progression to cervical carcinoma and in decreasing false-negative cytological screenings [242]. The telomeres are protective ends of the chromosomes. In normal human cells, telomeres become shorter when the cell divides. In 90% of human tumours telomerase is activated and synthesize telomeric sequences leading to maintenance of the telomeres and immortality [243, 244]. This is probably contributory to the steadily increasing tolerance for chromosomal changes with tumour growth [244].

PROTEOMICS

After the human genome sequence has been determined the interest moved to the analysis of the protein complement of the genome (proteomics) [224]. The term proteome was first coined by Wilkins et al 1994 [245] and refers to all the proteins in a cell, tissue or organism. The correlation between DNA sequence and protein is low, reflecting alternate splicing as well as post-translational modification, which explains the existence of a huge number of different proteins.

Two-dimensional gel electrophoresis (2-DE/2D PAGE), introduced by O´Farrell and others in 1975 [246], is currently the most important method for studying differences in protein expression in various biological samples.

The power of the technique is the capacity of separate simultaneously thousands of known and unknown polypeptides. The complex protein mixtures are separated on the bases of two physicochemical properties: the isoelectric point (pI) of the proteins using different pH gradients and their relative molecular mass using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [246, 247] (Figure 6). Proteins are visualized in gels by staining them with dyes such as colloidal coomassie brilliant Blue G, silver staining and fluorescent stain [248].
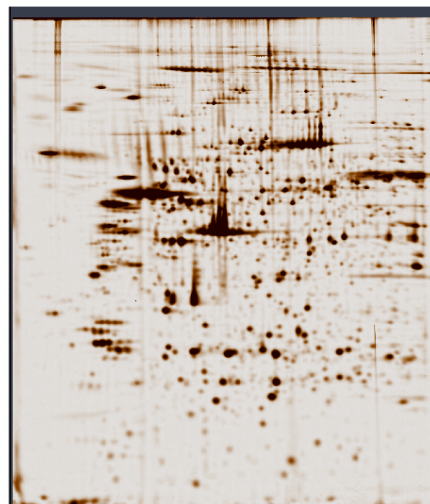

Figure 6. Representative 2DE-gel from normal vagina.

The 2-DE gels are scanned for computer assisted analysis, using software packages like PDQuest, Progenesis and Melanie, which include protein spot identification, quantification and gel-to-gel matching (evaluate tumour heterogeneity) [249, 250]. Protein identification is made by mass spectrometry (MS). The generated peptide masses are screened and mapped in protein databases (ex SwissProt, ExPASy). Several software programs for protein identification are available on line such as Mascot (http://www.matrixscience.com), which is dedicated to the analysis of protein sequences and structures as well as 2D PAGE.

The combination of 2D PAGE and MS is the most widely used technique for proteomics. The main limitations of the 2D PAGE are low resolution, low throughput and the large amount of samples required. Therefore competing automated high-throughput techniques are being developed, such as protein chips and liquid based separation. This might lead to a higher possibility to discover low abundance proteins, such as post-transcriptional modifications, that may have a role in tumourigenesis, but are difficult to detect with 2-DE.

By 2-DE studies significant differences in protein expression between tumour tissues and the corresponding normal tissues have been revealed for example in carcinoma of the colon, breast, lung, ovary, bladder and cervix [251-255]. In **paper V** the protein expression profiles of vaginal and cervical cancers have been compared.

With proteomic studies further characterization of proteins that are differentially expressed will provide a chance to identify tumour-specific diagnostic and prognostic markers.

AIMS OF THE THESIS

The main aims of this thesis were:

1. To get increased knowledge of vaginal carcinogenesis.
2. To get increased knowledge of prognostic variables (histopathological, clinical and biological) in PCV.
3. To get increased knowledge of epidemiological and etiological factors.
4. To assess similarities and differences between PCV and cervical carcinoma.

To reach these aims the following studies have been performed:

I. Assess the epidemiological, etiological and clinical data in relation to the age at diagnosis of PCV.

II. Evaluate the histopathological and clinical data in relation to disease-specific survival in PCV.

III. Investigate the laminin-5γ2 chain expression in relation to disease-specific survival in PCV.

IV. Analyse the pattern of genomic alterations in PCV by using CGH and study the cytogenetic data in relation to the presence of human papilloma virus genomes, the nuclear DNA content, the expression of laminin-5 and markers for proliferative activity and mutated p53.

V. Analyse the protein expression profiles by cluster analysis in PCV and to compare the protein pattern in PCV and cervical carcinoma in order to find a useful tool for correct classification and for increased biological knowledge.

MATERIAL AND METHODS

PATIENT MATERIAL

Paper I
A retrospective study of 341 cases of primary vaginal carcinoma diagnosed between January 1956 – December 1996 and treated at The Department of Gynaecologic Oncology, Radiumhemmet, Karolinska University Hospital.

Paper II
A retrospective study of 314 patients on the same material as in paper I with the exception that only squamous cell carcinomas were included.

Paper III
This study is based on 59 cases of primary vaginal malignancies diagnosed 1978-1990 and 1992-1995, treated at the Department of Gynaecologic Oncology, Radiumhemmet, Karolinska University Hospital.
The material was subdivided into two groups: 37 patients with poor prognosis (dying within ≤ 2 years) and 22 patients with good prognosis (surviving ≥ 8 years).

Paper IV
Stored paraffin-embedded tumour specimens were collected from 16 patients with primary vaginal carcinoma diagnosed and treated at the Department of Gynaecologic Oncology, Radiumhemmet, Karolinska University Hospital 1984-1993. CGH, DNA ploidy, immunohistochemistral analysis (p53, p21, cyclin A, Ki 67, laminin-5) and HPV typing were performed.

Paper V
Two-dimensional gel electrophoresis was performed on sixteen fresh frozen specimens from normal vaginal tissue (5 samples), primary vaginal carcinoma (6) and cervical carcinoma (5).

METHODS

Histopathology

The diagnosis was confirmed histologically by biopsy in all cases. The specimens were fixed in buffered formaldehyde, paraffin-embedded and diagnosed on haematoxylin and eosin (HE) stained tissue sections. All the histopathological slides were reviewed microscopically by one pathologist (Claes Silfverswärd) at the Department of Pathology, Karolinska University Hospital and by one of the authors (Kristina Hellman). Patients with a history of cervical or vulvar carcinoma within 10 years before diagnosis of PCV were excluded. This limit was chosen in order to minimise the risk of including metastases from former cervical or vulvar cancer.
In paper V the histopathology of the fresh frozen material was re-examined by Gert Auer and Ayodele Alaiya.

Stage

The patients were staged retrospectively according to the criteria of FIGO (1995) with the exception of patients with stage II tumours that were subdivided into stage IIA (subvaginal extension) and stage IIB (parametrial extension) [256]. Inguinal node metastases were judged as stage IVA.

Immunohistochemical methods

Stored paraffin-embedded specimens from vaginal carcinoma were used for the IHC analysis. Sections with a thickness of 4μm were cut from each specimen and put onto special treated slides (Menzel Superfrost plus) for the IHC studies. Sections for HE were cut before and after each section to confirm the diagnosis.

The original diagnosis as well as the representativity of the sections used for histochemical studies was reviewed by one pathologist (C.S.). The histopathologist had no knowledge of the results from the immunohistochemistry when reevaluating the sections. The immunohistochemical reactions were evaluated and visually scored by three of the authors (Hellman, Habermann, Auer) working independently of each other. Preparation and characterization of polyclonal antibodies raised in rabbit against a fusion protein containing the C-terminus of the laminin γ2 chain (containing amino acid residues # 1017-1178) and glutathione-S-transferase (GST) were performed according to methods described earlier [206, 218].

Laminin-5γ2 chain (paper III-IV)

Immunohistohemistry was performed using the standard horseradish peroxidase avidin-biotin-complex (ABC) technique (Vector, Elite Standard Kit, cat.PK-6100, Vector Laboratories, Inc., Burlingame, CA, USA).

The sections were first deparaffinized, rehydrated and microwave treated in 0,01 M sodium citrate buffer (pH 6) for 10 minutes at 500W. After rinsing in Tris buffered saline (TBS), pH 7.6, the endogenous peroxidase activity was blocked by immersion of the slides in 0,5% hydrogen peroxide in distilled water for 30 minutes and unspecific staining was prevented by using 1% bovine serum albumin (BSA) in TBS for 20 minutes. After incubation overnight at 4°C with the rabbit γ2 chain antibodies diluted in 1:200 in 1% BSA (2μg/ml), a biotinylated antirabbit IgG (diluted 1:200) was applied for 30 minutes, followed by 30 minutes incubation in the peroxidase avidin-biotin-complex. The peroxidase reaction was developed by using diaminobenzidine tetrahydro-chloride, 0.6 mg/ml with 0.03% H_2O_2 for 6 minutes. Between each step the slides were rinsed with TBS. After counterstaining with Mayers hematoxylin, the slides were dehydrated and mounted with a xylene-soluble mounting medium. As a control of the specificity of the method, the laminin-5γ2 chain antibody was replaced with BSA and the same procedure was performed on adjacent sections. A cervical cancer specimen positive for laminin-5γ2 chain served as a positive control.

Evaluation of the laminin-5γ2 chain immunohistochemistry

Only cells with distinct cytoplasmic immunoreaction were regarded as laminin-5γ2 chain positive. To declare a lesion positive, >1% of the cells had to show this specific immunostaining. To evaluate the extent of the immunoreactivity, the number of stained cells were estimated visually and categorized as follows: negative (≤ 1% of the tumour cells positive), 1+ (>1% to ≤5% of the tumour cells positive), 2+ (>5% to ≤30% per cent of the tumour cells positive) and 3+ (>30% of the tumour cells positive).

MIB-1, cyclin A, p53 (DO-1), p21(WAF-1) (paper IV)

All slides were deparaffinized with xylene, rehydrated and microwaved at 500W for 2x5 min in 10mM citrate buffer, pH 6.0. Intrinsic peroxidase activity was blocked with 3% hydrogen peroxide in methanol, followed by incubation with horse serum (1:20 dilution) in 0.1M PBS, pH6.0. The levels of protein expression were revealed by overnight incubation with the respective antibodies (see below) diluted in 1% (weight/volume) bovine serum albumin and visualized by standard avidin biotin-peroxidase complex technique (Vector Laboratories, Burlingame, CA). The following antibodies were used. The respective dilutions and suppliers are indicated in parentheses (all slides were coded and scored as reported previously [229, 257]):

1. The monoclonal antibody MIB-1 (1:150, Immunotech S.A., Marseille, France) was used for detection of the Ki-67 antigen

2. A monoclonal mouse antibody was used for detection of cyclin A expression (1:100, Novocastra Laboratories, Newcastle-upon-Tyne, UK).

3. The monoclonal mouse antibody DO1 (1:100 Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used for detection of p53 expression.

4. The monoclonal mouse antibody WAF-1 (1:15, Calbiochem, San Diego, CA) was used for detection of p21 expression.

Evaluation of the immunoreactivity of the antibodies above: Only cells with distinct brown staining confined to the cell nuclei were regarded as immunoreactive. The extent of the immunoreactive cells was assessed as the percentage of the positively stained cells in relation to the total number of cells. Overexpression of p53 implied that >50% of the cells were immunoreactive. Low expression and high expression of p21 was considered when <10% and ≥10% of the cells were positive respectively. Low and high expression of cyclin A meant that <10% and ≥10% were positive respectively. Concerning MIB-1, low and high expression was considered when <20% and ≥20% of the cells were positive respectively.

Comparative genomic hybridization (paper IV)

Tumour samples

The histopathological material was collected from biopsies that were taken before radiation therapy and surgery. The tumours were diagnosed on HE-stained tissue sections at the Department of Oncology and Pathology, Karolinska Institute and Hospital, Stockholm, Sweden. Ten sections were prepared from each tumour and used for histological diagnosis, immunohistochemistry (thickness: 4 µm), DNA-ploidy measurements (8 µm), and microdissection and DNA extraction (50 µm). A second HE-stained section was prepared subsequent to the sections for CGH analysis, and the histological diagnosis was confirmed in all cases. All data were obtained from the dissected areas.

CGH

Formalin fixed and paraffin embedded tumour samples were provided in 50 µm thick tissue sections. Normal control DNA was prepared from peripheral blood lymphocytes of a cytogenetically normal male. The tumour tissue was incubated in xylene (3x5min), followed by washes in 95% ethanol. According to the subsequent HE sections the deparaffinized tissue sections were microdissected in order to obtain representative tissue containing at least 80% of cancer cells. The microdissected cells were placed into

centrifuge tubes containing 95% EtOH. After centrifugation, the samples were dried and resuspended in 1 ml NaSCN (1M) and incubated overnight at 37°C. DNA was prepared using high salt extraction and phenol purification and labeled by nick-translation using biotin-11-dUTP (Boehringer Mannheim, Indianapolis, IN). Normal DNA was labeled with digoxigenin-12-dUTP (Boehringer Mannheim). Hybridization was performed on karyotypically normal metaphase chromosomes using an excess of Cot1-DNA (Gibco-BRL, Gaithersburg, MD). The biotin-labeled tumour genome was visualized with avidin-FITC, green fluorescence, (Vector Laboratories, Burlingame, CA) and the digoxigenin labeled reference DNA were detected with a mouse-derived antibody against digoxigenin followed by a secondary rhodamine conjugated anti mouse antibody, red fluorescence, (Sigma-Aldrich, Milwaukee, WI). Quantitative fluorescence imaging and CGH analysis was performed using Leica Q-CGH software (Leica Imaging Systems, Cambridge, UK).

If chromosomes or chromosomal subregions are present in identical copy number in both the reference and the tumour genome, the observed fluorescence is a blend of an equal contribution of red and green fluorescence. If chromosomes are lost or subchromosomal regions deleted in the tumour genome, the resulting colour is shifted to red. A gain of a certain chromosome in the tumour would be reflected by a more intense green staining on the respective chromosome in the reference metaphase preparation. Using computer software, the painted chromosomes are segmented and the fluorescence values determined on a pixel to pixel bases. The final step in a quantitative fluorescence measurement includes the calculation of average ratio profiles along the chromosomal axis. Values of 1 indicate equal copy numbers of the respective chromosomes in the tumour and test genome, a ratio of 0.5 a deletion of one homologous chromosome and ration of 1.5 reflect a trisomy in the tumour. Gene amplifications can be mapped to the reference metaphase chromosomes according to peak fluorescence ratios of more than 2.5.

Nuclear DNA content (paper IV)

Image cytometry was performed on Feulgen-stained histological sections (8 µm). The staining procedure, internal standardization, and tumour cell selection were based on methods described previously [258-260]. The DNA distribution profiles (histograms) were sampled from at least 100 structurally identified neoplastic interface cell nuclei from each specimen. All DNA-values were expressed in relation to the corresponding staining controls which were given the value 2c, denoting the normal diploid DNA-content. The computer software was ACAS 6.0, Ahrens ICM Cytometry System.

DNA content was expressed in c-units when comparing the DNA values of different tumours. As control cells normal human epithelial cells and normal human lymphocytes were assessed and their median value (P50) was defined as 2c.

The classification of the nuclear DNA histograms by image cytometry are usually subdivided into four types according to Auer et al 1980 [258].

Type I (diploid): The histograms are characterized by a single peak in the diploid or near-diploid region (1.8c–2.2c). The total number of cells with DNA values exceeding the diploid region (>2.2c) was <10%.

Type II (diploid-tetraploid): The histograms showed a single peak in the tetraploid region (3.8c–4.2c) or peaks in both the diploid and tetraploid regions (>90% of the total

cell population). The number of cells with DNA values between the diploid and tetraploid region and those exceeding the tetraploid region (>4.2c) was <10%.

Type III (proliferating diploid): The histograms represented highly proliferating near-diploid cell populations and were characterized by DNA values ranging between the diploid and the tetraploid region. Only a few cells (<5%) showed more than 4.2c.

The DNAhistograms of types I, II and III thus characterize euploid cell populations.

Type IV (distinctly aneuploid): The histograms showed increased (>5%) and/or distinctly scattered DNA values exceeding the tetraploid region (>4.2c). These histograms characterize aneuploid populations of interphase nuclei with decreased genomic stability.

The specimens in this material were divided into two main groups: (i) diploid cases with a distinct peak in the normal 2c region and no cells exceeding 5c, (ii) aneuploid cases with a main peak around the 4c region and varying numbers of cells (>5%) exceeding 5c.

HPV Genotyping (paper IV)

In the purified DNA samples the HPV genomes were identified with the line blot assay using PGMY primers. In this assay, amplified products are tested against 27 HPV type specific probes, which are immobilized on a filter strip [261]. The 27 probes include high risk HPVs (such as 16, 18, 31, and 45). Beta-globulin amplification was used as a positive control to evaluate the adequacy of tumour DNA.

Two-dimensional gel electrophoresis (2-DE)

Sample preparation

Sixteen tissue biopsies (about 3mm x 3mm) were analyzed consisting of 5 biopsies from normal vaginal epithelium, 6 from primary vaginal carcinomas and 5 from primary cervical carcinomas.

The eleven tumour biopsies were taken from patients with histopathologically confirmed diagnosis of either vaginal or cervical cancers.

The 5 normal vaginal biopsies were obtained from the upper part of the vagina, approximately 1 cm from the vaginal fornix in postmenopausal women undergoing total hysterectomy for either benign disease or endometrial/ovarian carcinoma. Each tissue sample was macroscopically and microscopically examined by three of the authors (Hellman, Alaiya and Auer) and only representative, non-necrotic tissue samples were used.

All the tissue samples were prepared according to a frozen tissue preparation method [262], with slight modification. Briefly, whole tissue biopsies were kept frozen in liquid nitrogen and mechanically homogenized using a pestle and mortar. Each sample was then dissolved in 300 – 500 µl lysis buffer containing 7M urea, 2M thiourea, 4% SDS, reducing agents and protease inhibitors.

Protein concentration was determined using the Bradford method [263].

Electrophoresis, scanning and image analysis

The 2-DE gel electrophoresis was done according to Franzen 1993 and Okuzawa 1994 [264, 265].

1. Separation of the polypeptides by isoelectric focusing (first dimension). For each sample, the equivalent of 100 µg total solubilized proteins was dissolved in 350 µl volume of rehydration buffer (2% (v/v) IPG-buffer 4-7 linear) was loaded onto a 17 cm

IPG-strip 4-7 linear (Bio-Rad). This gives better resolution and better overview of protein spots across the entire chosen pH window. In addition, the linear gradient also gives a better estimation of the isoelectric point (pI). Isoelectric focusing was performed overnight for each individual sample to a total of 45.5 kVh using Bio-Rad IEF unit (200C). Before the second dimension the strips were finally shortly equilibrated in two steps (15 minutes each) with iodoacetamide (2.5%) in the first and DDT in the second.

2. Separation of the polypeptides by SDS-PAGE (second dimension). 10-13% gradient SDS gels were used to carry out the second dimension. The IEF strips were applied on the top of the gels, sealed with 0.5% agarose containing electrophoresis running buffer and subjected to electrophoresis overnight.

The proteins were visualized by silver staining [266] (Figure 6).

After electrophoresis and staining, only high quality gels were used. Occasionally, some samples had to be rerun in order to obtain comparable quality with other 2-DE gels. Stained gels were scanned at 100 μm resolution using a laser densitometer, and data were analyzed using the PDQUEST™ software (version 7.1.0, Bio-Rad). Gel images were compared for qualitative and quantitative differences. Polypeptide quantities were calculated in ppm of the total integrated optical density.

Mass spectrometry

Protein spots with statistically significant variability in the expression pattern between normal vaginal epithelium, cervical and vaginal cancers were selected for identification by mass spectrometry.

Micropreparative gels for protein identification were prepared essentially like the analytical gels, except that larger amounts (750μg) of total proteins were loaded and subjected to isoelectric focusing. Following 2nd dimension electrophoresis, gels were stained using Coomassie colloidal stain. The 2-DE gels were analyzed by PDQUEST software and spots of interest were manually excised using a clean sharp scalpel and transferred into an eppendorf tube. In-gel digestion for peptide mass fingerprint analysis was done manually with trypsin, and digests were desalted using Zip Tip (Millipore) as recommended by the manufacturer. Peptides were diluted in 70% acetonitrile/5% formic acid and mixed 1:1 (v/v) with a saturated matrix solution containing α-cyano-4-hydroxycinnamic acid in 30% acetonitrile/0.1% trifluoroacetic acid. Mass mapping of tryptic peptides was performed using MALDI-TOF (above protocol) or Cap-LC-MS/MS on Micromass Q-TOF Ultima mass spectrometer with LC-packings pep Map C18, 75μm ID column using a gradient of 7-80% (95% acetonitrile and 0.1% formic acid) over a period of 35 minutes.

Trypsin fragments of masses 842.50 Da and 2211.10 Da were used as internal standards for spectra calibration. Data generated were screened in databases using a mass tolerance ≤20 ppm. The licensed ProteinLynx™ Software (Micromass) or mascot was used for mass mapping [125].

The above protocol of MALDI-TOF analysis has a sensitivity of femtomole amounts of standard 2-DE gel separated proteins. For a positive identification of the peptide mass fingerprinting, protein scores greater than 72 were considered significant (p<0.05), as calculated by the MASCOT scoring algorithm. In addition, at least four matching peptides should be found and more than 50% of the measured masses should match the theoretical peptide fragments.

Data analysis

Both quantitative and qualitative 2-DE data sets were generated from PDQUEST, a 2-DE software analysis program. The data set generated from the matchset based on each individual sample was imported into J-Express as an Excel test format in the form of a data table, with rows representing gels and columns representing spots [254]. The pre-processed data were analyzed by hierarchical clustering [267-269] using the J Express pro software v 2.1 [63]. The J-Express program was primarily designed to analyze microarray data but equally accepts data sets generated from 2-DE analysis.

Hierarchical cluster analysis

Hierarchical cluster analysis is a statistical method that is based on measured variables capable of identifying relatively similar groups of samples. This method is based on the strong assumption that an appropriate distance measure for comparing cases has been carefully selected. Thus, the outcome of the clustering analysis depends on the method of calculation of the distance between samples being compared.

In this study the degree of similarity was calculated using the Bray Curtis distance metric and a complete linkage clustering method. The clustering patterns are then represented diagrammatically as dendrograms with trees and branches depicting the degree of sample relatedness.

Correspondence analysis

We have used correspondence analysis to further evaluate the same datasets used in hierarchical cluster analysis. This was considered as a means to test if the observed set of genes can indeed discriminate the sample groups, bearing in mind the small sample size of this study.

Correspondence analysis (CA) is a computational method that is similar to principal component analysis (PCA) with potential to study association between groups of samples based on selected variables.

The data being subjected to CA is presented as two-dimensional graphical display. This method is capable of visualizing different structures within a complex dataset.

The principle behind the CA is an attempt to group together objects that are similar while dissimilar objects are separated off. The degree of similarity or difference is measured by distances between objects or groups of objects. The analysis has been used to evaluate different complex microarray data [270].

Statistics

In paper I the dependent variable was age at diagnosis and in paper II-III the dependent variable was disease-free survival.

Paper I

In order to evaluate the difference in age at diagnosis, the analysis of variance (ANOVA) was used to test the difference between means of individual groups both in the univariate and in the multivariate analysis. Age at diagnosis was the dependent variable and histopathological, clinical and epidemiological characteristics were independent variables (Tables 2 and 3). Five variables (age at menopause, pregnancies, prior hysterectomy, earlier dysplasia and earlier irradiation) were only evaluated if they

had occurred before 50 years of age. The 50-year limit was chosen because of distributional reasons in order to include as many patients as possible that could be evaluated with respect to the different variables. Only patients with age at diagnosis of PCV ≥50 years were considered with respect to these variables thereby avoiding the pitfall of overlapping exposure and follow-up periods [271]. The exposure period is thus the period before 50 years of age and the follow-up period is the period from the age of 50 years until the diagnosis of PCV.

The variable menopause age was split up at 46 years in order to find a correlation between age at diagnosis and early menopause. The "menstruation span" was calculated as: age at menopause minus age at menarche minus the number of births multiplied by 1.5 in order to take into account the amenorrhea during child-bearing and lactation [272].

Paper II

Differences in distributions between groups were compared with the chi-square test. Survival data was evaluated with the life table method taking censored observations into account. Differences in survival between groups were tested with the Gehan-Wilcoxon statistic (categorical factors). Continuous variables and multivariate analyses were performed with Cox regression.

Paper III

To evaluate the difference between short- and long-time survivors univariate and multivariate analysis were performed. The univariate analysis was carried out with chi-square test for the categorical variables and with T-test for the continuous variables. The multivariate analysis were performed with logistic regression.

The analysis of variance (ANOVA) was used to test the difference between means of individual groups.

Paper IV

In order to detect the relationship of cytogenetic imbalances, expression levels of immunohistochemical markers, the HPV status, and the clinical course, data were dichotomized and analyzed by Fisher's exact test at a level of $\alpha = 0.05$. Kaplan-Meier survival curves were estimated and compared by log rank test.

Paper V

The sets of genes used in the cluster analysis were selected using Student's t-test and the Mann Whitney ranked test analysis (P<0.05) between normal vaginal tissue and vaginal cancer samples. A similar analysis was made between groups of primary vaginal cancer and primary cervical cancer. These variables were then used for the classification of the samples into different groups.

Ethics

All studies were approved by the Northern Research Ethics Committee at the Karolinska Hospital.

RESULTS

PAPER I - FACTORS INFLUENCING THE AGE AT DIAGNOSIS

Epidemiological, histopathological and clinical variables were studied retrospectively on 341 cases of primary vaginal carcinoma and the relation to patients' age at diagnosis was evaluated. The mean age at diagnosis was 67 years (range 22-93) and it has increased significantly during the years (p<0.001). The mean age at menarche and natural menopause was 14.1 and 48.9 years respectively. The vast majority of the patients were postmenopausal (91%). Age at menarche showed a negative significant correlation with year at birth (p<0.001), (Figure 7).

Nulliparity and parity ≥4 were found in 22% and 20% of the cases respectively compared with 12% and 9% in the same age-group in the Swedish normal population. Smoking and a history of being divorced were also more common among the patients in the study than in the Swedish normal population.

A background history of gynaecological malignancies was found in 13% of the patients, pelvic irradiation therapy in 14%, cervical dysplasia in 14% (Figure 8), hysterectomy in 23% and gynaecological infections in 7% of the cases.

The univariate statistical analysis showed that younger age at diagnosis significantly correlated with a history of cervical dysplasia (p=0.001), hysterectomy (p=0.014), gynaecological infections (p=0.001) and tumours located in the upper part of the vagina (p=0.008), whereas older age at diagnosis significantly correlated with late menarche (p<0.001) and exophytically growing tumours (p=0.021). Parity, nulliparity, age at menopause, menstruation span, prior irradiation, histology, grade, tumour size and stage had no influence on age at diagnosis in the statistical analysis. In the multivariate regression analysis the remaining independent predictors were a history of cervical dysplasia (p=0.006) and age at menarche (p=0.022).

Summary

Several variables correlated with age at diagnosis, but in the multivariate analysis the remaining independent predictor for young age at diagnosis was prior cervical dysplasia and for old age at diagnosis late menarche. Parity ≥4 as well as nulliparity, smoking and unstable marital status were more common among patients with PCV than in the general Swedish female population.
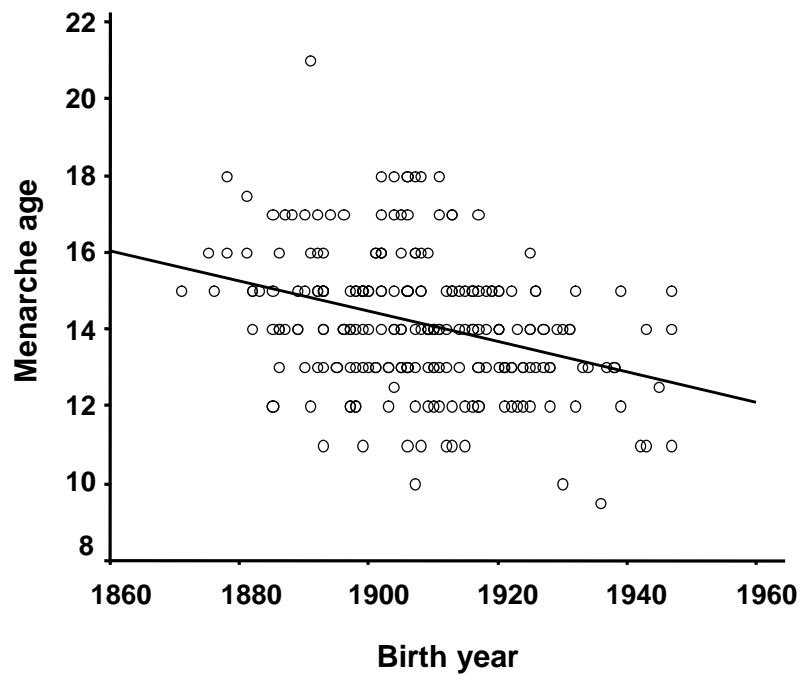
Figure 7. PCV. Correlation between year of birth and age at menarche (p<0.001).
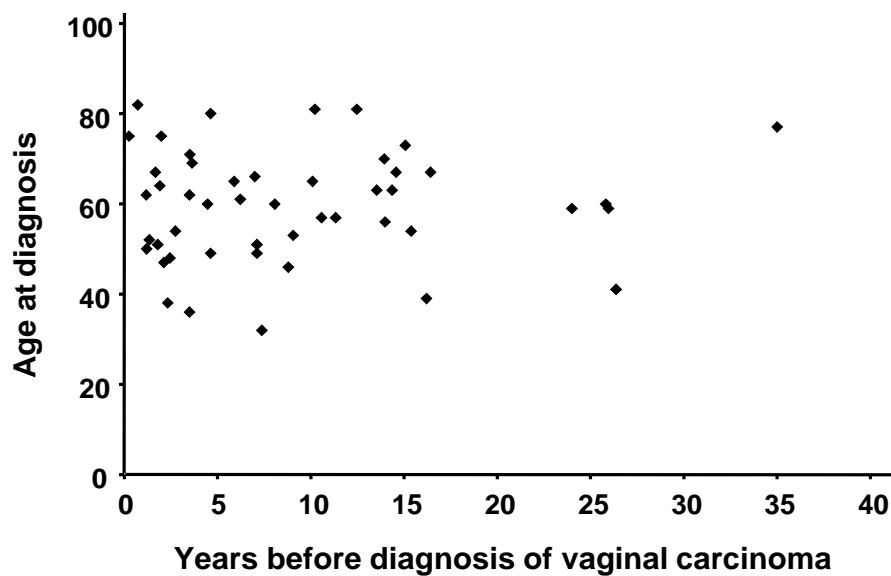

Figure 8. Patients with PCV and a history of cervical dysplasia (n=48). Age at diagnosis of patients with primary vaginal carcinoma and interval from the dysplasia to the carcinoma. The vaginal carcinoma was diagnosed with a mean of nine years (range 3 months-35years) after a previous cervical dysplasia. Each dot represents one case.

PAPER II - HISTOPATHOLOGICAL AND CLINICAL PROGNOSTIC FACTORS

The impact of clinical and histopathological factors on disease-free survival was evaluated in this retrospective study comprising 314 patients of primary vaginal squamous cell carcinoma diagnosed and treated 1956-96 at Radiumhemmet. The diagnosis in all cases was confirmed histologically and reevaluated by an experienced pathologist. The overall disease-specific survival rate (censoring non-cancer-related deaths) at 5 and 10 years was 45% and 39% respectively. For DFS in each stage see Table 3. In stage I the 5-year survival rate was superior (75%). In stage IVb all patients died within 9 months.

Table 3. Primary Carcinoma of the Vagina.
Cause-specific survival rate by stage.

Stage	Survival rate (%)				
	5-year	10-year	15-year	20-year	25-year
I	75	68	68	63	63
IIa	36	30	30	30	30
IIb	35	28	28	28	28
III	34	30	30	30	25
IVa	20	13	6	0	
IVb	0				

Significantly more patients were diagnosed with early stage disease in the later part of the study period (Table 9). However there was no significant difference in survival if diagnosis of PCV was made between 1956-75 or 1976-96. Location in the upper third or in the entire vagina was most common. In hysterectomised patients PCV was significantly more frequently found in the upper vagina. Vaginal bleeding was the presenting symptom in the majority of the cases. Eight percent of all the cases had inguinal lymph node involvement at diagnosis and 16% of those with tumour located to the lower third or the entire vagina. Distant metastases were found in only 2% at diagnosis.

The univariate statistical analysis demonstrated that several factors correlated significantly with shorter survival (ulcerating tumours, large tumours ≥4cm, advanced stages, tumours comprising more than one-third or one wall, growth into the rectovaginal septum, inguinal lymph node metastasis, symptoms at diagnosis and high age at diagnosis). Patients treated with surgery only or in combination with BT and/or ERT had significantly better survival compared to those not treated with surgery (p=0.025). The majority of the patients treated with surgery were in stage I. When looking at stage I separately there was, however, no significant difference in survival between the different treatment modalities. There was no significant difference in survival between patients in the whole material treated with BT only and those treated with combination therapy (ERT+BT). Tumour grade and other histopathological features did not correlate significantly with survival. In the multivariate analysis the remaining independent predictors for disease-specific survival were stage (p<0.001), tumour size (p<0.001) and age at diagnosis (p=0.004).

PAPER III - LAMININ-5ɢ2 CHAIN EXPRESSION AND PROGNOSIS

The expression of laminin-5γ2 chain in primary malignancies of the vagina and the correlation of the γ2 chain expression with the disease-free survival were investigated. The material consisted of 56 cases of primary vaginal carcinomas (and three non-epithelial tumours), subdivided into short- (≤2 years) and long- (≥8 years) time survivors (Table 4).

Table 4. Laminin γ2 chain immunoreactivity in vaginal carcinoma and clinical and histopathological data among short- and long-time survivors (n=56).

	Survival ≤2 years (n=34)		Survival ≥8years n=(22)	
	n	%	n	%
Laminin ɢ2 chain immunoreactivity				
1+ and 2+	17	50	17	77
3+	17	50	5	23
Stage:				
I	9	26	17	77
II-IV	25	74	5	23
Degree of differentiation (n=55)				
Well	7	21	2	9
Moderate	19	56	10	45
Poor	8	23	9	41
Histopathology:				
Squamous	33	97	19	86
Adeno			2	
Small cell	1		1	

Paraffin-embedded tumour material was stained immunohistochemically with a polyclonal rabbit antibody (a fusion protein containing the C-terminus of the laminin-5γ2 chain and Glutathione-S-transferase).

Immunopositivity was noted for all invasive vaginal tumours of epithelial origin, while the three non-epithelial tumours were negative (Table 5).

Laminin-5γ2 chain expression showed a positive significant correlation with tumour size (p=0.003), while no correlation was found with clinical stage, patients age at diagnosis and grade. Among the cases with high laminin-5γ2 chain expressions the majority were short time survivors (77%). In the univariate analysis following parameters correlated significantly with disease-free survival: laminin γ2 chain immunoreactivity (p=0.041), clinical stage (p<0.001), patients age at diagnosis (p<0.001) and tumour size (p<0.001). In the multivariate analysis however, only tumour size and patients age independently could explain the variation in survival.

Summary

All vaginal tumours of epithelial origin showed immunoreactivity for the γ2 chain of laminin-5. A positive significant correlation was found between γ2 chain expression and tumour size. In the univariate survival analysis expression of laminin-5γ2 chain was indicative of poor outcome, but not in the multivariate analysis.

PAPER IV – GENOMICS. A RECURRENT GAIN OF CHROMOSOME ARM 3Q IN PRIMARY SQUAMOUS CARCINOMA OF THE VAGINA

On 16 paraffin-embedded tumour specimens from PCV CGH-analysis, DNA-ploidy, immunohistochemistral analysis (MIB-1, cyclin A, WAF-1, p53 and laminin-5) and HPV-typing were performed. All cases except one showed a highly aneuploid distribution of the DNA content. The most common DNA gain was mapped to chromosome arm 3q in 69% of the cases Other frequent gains were localised to 19p (50%), 5p (50%), 6q and 19q (44%), 1q and 17p (38%), 1p, 7p, 14, 16q, 18p, and 22 (31%), 2p, 9q, 12, and 18q (25%) (Figure 9). Chromosomal losses were infrequent and mapped to chromosome arms 13q (2x), 8p (1x), and 9p (1x). The average number of copy alterations (ANCA) amounted to 7.5 for the autosomes. The presence of HPV genomes was analyzed by a line blot assay in the same DNA that was used for CGH. Eight samples could be analyzed and two of these were positive for high-risk HPVs (16 and 52). The immunohistochemistral studies revealed that the markers of proliferative activity were increased in the majority of the cases (cyclin A and MIB-1 in 63% and 88% respectively).

Increased expression of the tumour suppressor gene p53 was observed in only two samples. In one of these cases
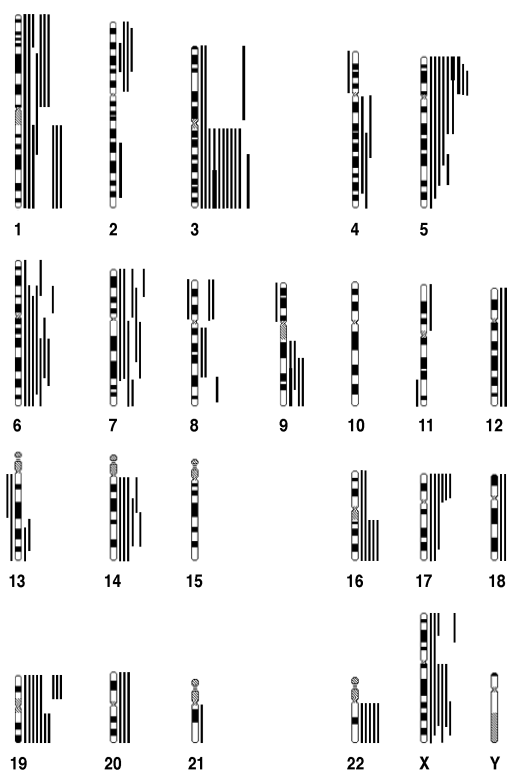

Figure 9. Summary of genetic imbalances in 16 specimens of PCV. Bars on the left side of the chromosome denote a loss of sequence of the tumor genome, bars on the right side indicate gain and bold bars indicate high level copy number increases (amplifications).

the p21/ WAF1 expression levels were low, which is indicative of mutational inactivation of p53. In general the expression levels of WAF1 were increased (94% of the samples). The expression of laminin-5γ2 chain was elevated in 69% of all samples. In the statistical evaluation patients older than 70 years at diagnosis (p=0.031), with a tumour size greater than 4 cm (p=0.025) and an increased laminin-5γ2 chain expression (p=0.006) had a significantly shorter survival time. Histology, location of the tumour, duration of the disease, history of previous malignancies or hysterectomy had no influence on survival. Patients with a history of CIN showed longer survival than all other patients (p=0.045). Samples that were negative for HPV had significantly more often higher ANCA-value (p=0.031) than samples positive for HPV.

Summary

Vaginal carcinomas are defined by a specific distribution of chromosomal aneuploidies with 70% carrying relative copy number increases that map to chromosome arm 3q.

Age at diagnosis, tumour size and laminin-5γ2 chain expression showed a significant negative correlation with survival in a univariate analysis.

PAPER V – PROTEOMICS. PROTEIN EXPRESSION PATTERNS IN PRIMARY CARCINOMA OF THE VAGINA

The protein expression profiles in PCV, normal vaginal tissue and primary cervical carcinoma were examined in this study by using two-dimensional polyacrylamide gel electrophoresis (2-DE). The goal was to compare the protein profiles of the three tissue specimens in order to point out similarities or differences for diagnosis.

Sixteen fresh-frozen tissue samples were analyzed (six samples from PCV, five from normal vaginal tissue and five from primary cervical cancers). An average total number of 1373 spots were resolved on the 2-DE gels and between 75 – 82% of the spots were matched between all the gels.

Correlation analysis of the 2-DE gels was performed to analyze the heterogeneity in protein expression between the match-sets. A high correlation was seen between pairs of normal vaginal tissue, pairs of PCV and pairs of cervical cancer with correlation coefficients (r) of 0.70, 0.76 and 0.79 respectively. A high correlation was also observed between pairs of vaginal and cervical cancers (r =0.68), while there was a lower correlation between the samples of PCV and normal vaginal tissue (r=0.62) and between cervical cancer and normal vaginal tissue (r=0.55).

Quantitative and qualitative changes in the protein expression were generated from PDQUEST. Between pairs of 2-DE gels from only vaginal and cervical cancers 23 protein spots were significantly differentially expressed by using both the Mann Whitney and the t-test. Both hierarchical cluster analysis and two-dimensional correspondence analysis could correctly classify the samples in three distinct groups (normal tissue, vaginal and cervical cancer) by using this set of only 23 proteins.

Protein spots with statistically significant variability in the expression pattern between the three tissue specimens were selected for identification. Some of these proteins were identified through matching with 2-DE maps of proteins already identified, using bench top MALDI-TOF MS. Among the protein spots so far identified are high molecular weight tropomyosin 1, cytokeratins 5, 15 and 17, apolipoprotein A1, annexin V, GST, calreticulin, HSP 27 and HSP 70.

Summary

Hierarchical cluster analysis of the proteomics data from specimen of normal vaginal tissue, vaginal and cervical cancers allowed clear discrimination between the three groups.

DISCUSSION

Due to the rarity of PCV the established knowledge originates from studies based on small materials. Consequently little is known about genesis, natural history and factors predicting the prognosis. Thirty percent of patients with PCV have had prior CIN or invasive cervical carcinoma [75]. It is therefore plausible to assume a similar etiology and natural history as for cervical carcinoma. This hypothesis is supported by the fact that the cervix and vagina are exposed to the same carcinogenic agents, are lined with the same squamous cell epithelium and are related embryologically. However, tumours in these organs occur in different age-groups, cervical carcinoma predominantly at younger ages (50% of the cases <50 years old), while PCV occurs predominantly in postclimacteric women and only 10-15% are younger than 50 years at diagnosis [3, 55, 57]. Clinical stage has been pointed out as the most important prognostic variable for PCV by most studies. The material in this thesis (paper I and II) is the largest so far published on PCV and no prior studies have been conducted on either laminin-5 expression, proteomics or genomics. Against this background the main objectives of this thesis have been to increase the understanding in the genesis of PCV, etiology and prognostic factors.

The impact of epidemiological, histopathological and clinical variables on the age at diagnosis of PCV was studied in **paper I**. The hypothesis was that factors influencing the age at onset should be connected directly or indirectly with the genesis of the disease. The finding that age at diagnosis has increased during the study period probably only reflects the change in the age distribution of the population at risk.

In the univariate statistical analysis several variables correlated significantly with younger age at diagnosis (cervical dysplasia, hysterectomy, gynaecological infections and tumours located in the upper part of the vagina). Prior cervical dysplasia is frequently described in patients with PCV [35, 72, 75, 83, 91] and might be related to a common etiology (HPV). Likewise hysterectomy (prior to age of 40) has been associated with increased risk for PCV [49] and is possibly related to residual or occult dysplasia in the upper vagina [27, 75, 273].

Advanced age at diagnosis correlated significantly in the univariate analysis with late menarche and exophytically growing tumours. Late menarche and early menopause have been associated with an increased risk for PCV [49].

Other risk factors discussed in the literature, such as parity ≥4 as well as nulliparity, smoking and unstable marital status [37, 52, 56, 84] were considerably more common among patients with PCV than in the general Swedish female population [274-276].

In the multivariate analysis the remaining independent factors for young and advanced age at diagnosis were prior cervical dysplasia and late menarche respectively.

These results might imply that there are two different types of vaginal carcinoma occurring at separate ages and with different etiologies as described for vulvar carcinoma [277]. Patients that are younger at diagnosis of PCV often have a background history of cervical dysplasia why the disease in these cases could be etiologically related to cervical carcinoma maybe with the development over preinvasive stages and probably HPV-dependent [54, 278]. Furthermore, one study has shown HPV of different types in all VAIN lesions [33].

Vaginal carcinoma of advanced ages, which is the most common age-group, might rather be associated with hormonal factors and/or trauma to the vagina than HPV.

Hormonal factors such as the estrogen deficiency postmenopausally might play a role in the vaginal carcinogenesis. Hysterectomy with oophorectomy may fit into this hypothesis. The impairment of ovarian function through irradiation may also be of importance in addition to the carcinogenetic effect of ionising radiation. Due to the estrogen decline postmenopausally the vaginal epithelium becomes thinner and more sensible to injuries from surgery (hysterectomy, prolapse surgery) or from pessaries which could contribute to malignant transformation. Other conditions leading to a low estrogen lifetime exposure include high age at menarche, low age at menopause and nulliparity. Furthermore, a high estrogen lifetime exposure, for example multiparity, intake of exogenous estrogens and obesity, might have a protective effect. Grand multiparity might, on the contrary, increase the risk due to the repetitive trauma to the vagina.

There is clinical evidence that VAIN lesions in postmenopausal women should be treated with estrogens applicated vaginally as a first line treatment [100]. However it has been found that steroid hormones (both estrogen and progesterone) can induce malignant transformation in the squamous cell epithelium of the vagina and cervix by enhancing transcription of HPV E6/E7 oncoproteins [98].

Since HPV infection is not common in postmenopausal women the above mentioned synergism between HPV and steroid hormones for the squamous cell carcinogenesis preferably may occur in premenopausal women.

The survival rates for vaginal carcinoma have improved during the last decades mostly due to earlier detection and advances in the radiation technique [3]. In **paper II** we found that the survival rate in stage I was exceedingly superior compared to more advanced stages, which is in accordance with most other studies. Significantly more patients were diagnosed in stage I in the last 20 years of the study period. However, there was no difference in disease-specific survival related to diagnose year. A reason could be that older women suffer from PCV and that many stage I patients have large tumours. Location in the upper third of the vagina was most common with predominance for the posterior wall. In contrast to earlier studies no prognostic impact depending on vaginal location was found [83, 107, 279]. It was the extension of the tumour in the vagina, which reflects the tumour size, rather than the location that had prognostic importance in the univariate analysis. Location of the tumour to the lower third has been associated with worse prognosis probably due to early spread to the inguinal lymph nodes [94]. In agreement with most other studies vaginal bleeding was the most frequent symptom at detection. Lack of symptom was a significant indicator of superior survival, a finding also noticed by other investigators [107, 280], reflecting detection at earlier stage. Surgical treatment alone or in combination with RT yielded superior survival than RT alone, a finding in accordance with some studies [87, 124, 279], but not with others [106]. However, in stage I where surgical treatment is recommended [124], our material showed no difference in survival depending on type of treatment. It may be difficult to interpret the results as only a minority of the patients was treated with surgery.

The multivariate regression analysis revealed three remaining significant independent predictors of poor survival, advanced age at diagnosis, large tumours ($\geq$4cm) and advanced clinical stage, also demonstrated in other studies [83, 106, 128, 281].

Common background factors with no prognostic significance were prior hysterectomy, other gynaecological malignancies and pelvic irradiation which is consistent with earlier reports [75, 83, 87, 90, 91, 109].

The vaginal carcinoma appears preferentially to be locally invasive and distant metastases seem to be rare at first presentation. The prognosis for PCV is worst when the tumour is large and has grown beyond stage I. However, stage I lesions are quite heterogeneous concerning size and depth of growth why the treatment must be individually tailored, also taking into consideration other aspects such as patient age and general condition. A subdivision of stage I to IA and IB in the FIGO guidelines for staging would therefore be important.

The expression and prognostic impact of the epithelial basement protein, **laminin-5ɡ2** chain, was investigated in short- and long-time survivors of PCV. Prognostic importance of the protein has been reported in some earlier studies [209, 214]. To our knowledge laminin-5 expression has not been investigated in vaginal carcinoma. Immunopositivity for the γ2 chain was found in all cases of epithelial vaginal cancers but not in the non-epithelial ones which is consistent with the literature [204]. Strong expression of the laminin-5γ2 chain showed a slight significant correlation with poor survival in the univariate analysis, but not in the multivariate analysis where the only factors predicting poor outcome were large tumours and advanced age at diagnosis.
To further evaluate the prognostic and diagnostic importance of laminin-5γ2 chain larger studies also including precancerous stages are imperative.

Comparative genomic hybridization of PCV revealed a recurring pattern of chromosomal aneuploidies. The most frequent chromosomal aberrations were gains on chromosome arms 3q, 5p and 19p. The distribution is strikingly similar to the one in advanced cervical carcinomas where copy number increases on 3q, 19p and 5p occur in about 70%, 50% and 35% of the cases respectively [237, 238, 282]. This finding might support the hypothesis of similarities in tumourigenesis for vaginal and cervical carcinomas. Furthermore like in cervical carcinoma a negative immunoreactivity for p53 was found in the majority of the cases, concomitant with high expression of p21/WAF1. In cervical carcinoma it has been supposed that p53 is inactivated via the HPV E6 oncoprotein which circumvents the need for TP53-inactivating mutations. However, HPV is not as common in PCV as in cervical carcinoma where it can be detected in almost all cases [54, 283, 284]. In our study HPV was found in 2/8 cases, while other studies have reported an average of 50% [50, 51, 53, 67, 68]. The HPV-positive cases had a CGH profile that differed from the HPV-negative cases with fewer genomic imbalances. Given the few cases analysed successfully in our study it is difficult to draw any conclusions. In PCV p53 is probably inactivated partly in another manner than via HPV oncoprotein E6/E7 and mutations of TP53.
Copy number increases on chromosome arm 3q is frequent found also in carcinoma of the vulva, ovaries and the fallopian tube [239, 240, 285], but not in endometrial carcinoma [286]. In primary carcinoma of the fallopian tube all cases were negative for HPV and showed strong p53 immunoreactivity, suggesting TP53 inactivation by gene mutation [239]. It could also be observed that a gain on chromosome arm 3q occurs independently of the presence of HPV.
All PCV, but one, showed a highly aneuploid distribution of the nuclear DNA content. The vaginal tumour with diploid pattern was a small tumour diagnosed at an early stage due to regular gynaecological check-ups because of previous VAIN. Furthermore a high proliferative activity was observed in the majority of the cases.

The vaginal carcinoma appears to be a tumour of high genetic instability with similar chromosomal aberrations as in advanced cervical carcinoma suggesting it to be an aggressive rapidly growing tumour or alternatively detected at late stages of disease progression.

Two-dimensional gel electrophoresis (2-DE) has been used to examine the heterogeneity in gene expression in tissues from different malignancies. Significant differences in the polypeptide expression between several tumour tissues and the corresponding normal tissues have been demonstrated [251-253, 255, 262]. We used 2-DE to analyze the polypeptide expression pattern in samples from PCV, normal vaginal tissue and cervical carcinoma. Vaginal and cervical carcinoma seemed to be quite homogenous in their gene expression according to the correlation analysis (r=0.68). Studies from pairs of ovarian and pairs of breast carcinoma have shown large intertumoural heterogeneity respectively. PCV and cervical carcinoma appear consequently to be more homogenous, indicating similar etiology.

A total of 23 protein spots were significantly differentially expressed among cervical and vaginal carcinoma. By using this dataset of the 23 proteins in hierarchical cluster analysis and correspondence analysis all samples could be classified into three groups (normal vaginal tissue, vaginal- and cervical carcinoma). One case of PCV with prior cervical carcinoma 35 years ago was also correctly classified as vaginal carcinoma.

Cluster analysis is a method of describing similarity between samples based on their pattern of gene expression [268] and accurate classification of breast cancer tissues has been achieved [287]. In ovarian carcinoma cluster analysis of a set of differentially expressed proteins could be used as prognostic tool [267].

In the clinical situation it is sometimes difficult to discriminate between PCV and cervical carcinoma especially in patients with recurrent disease. Cluster analysis might thus be used for correct classification. There is further a possibility to find diagnostic and prognostic markers among the proteins that are differentially expressed. This may lead to novel techniques for treatments better adapted to the degree of malignancy of the tumour.

CONCLUSIONS

The results from the genomic, proteomic and retrospective studies support the hypothesis of a common etiology for cervical and vaginal carcinoma even though these diseases occur at different age-groups. The occurrence at different age-groups could be explained by the hypothesis that the response of these organs to the same carcinogenic stimulus (for example HPV) varies with age and that the rate of response of the cervix may be more rapid (perhaps due to the transformation zone) than the response rate of the vagina [55]. Alternatively there might be an additional carcinogenic stimuli predominantly occurring in the older age group (the estrogen deficiency?) to which the vagina is responsive. The survival rate of PCV has increased over the years, but despite earlier detection it is still quite low. This might indicate that vaginal carcinoma is an aggressive disease, which is supported by the results from the genomic study where high genomic instability, aneuploidy and high proliferative activity seemed to characterize PCV. The only variables that independently affected survival were crude clinical factors like stage, tumour size and age at diagnosis.

As in other malignancies early detection is crucial to provide better prognosis. Screening is not a suitable method due to the rarity of PCV. As vaginal lesions are easily detectable it is of importance to perform a thorough inspection of the vagina with a simple speculum investigation, especially in elderly women with common background factors for PCV, such as a history of hysterectomy, pelvic irradiation and/or earlier gynaecological malignancy and in patients with cervical or vaginal dysplasia.

Further studies are needed to identify biological prognostic markers to discriminate between low and high malignant tumours, where proteomics approach promises well.

ACKNOWLEDGEMENTS

REFERENCES

1.   The National Board of Health and Welfare. Cancer Incidence in Sweden 2003.

2.   Onkologiskt Centrum. Cancerincidens 1994-1999, Stockholm-Gotlandregionen 2001.

3.   Beller U, Maisonneuve P, Benedet JL, Heintz AP, Ngan HY, Pecorelli S et al. Carcinoma of the vagina. Int J Gynaecol Obstet 2003;83 Suppl 1:27-39.

4.   Pettersson F. Annual report on the results of treatment in gynecologic cancer. FIGO 1987-93. Stockholm: Radiumhemmet; 1999:22.

5.   Cruveilhier J. Cancer ulcéré de la paroi antérieure du vagin et du bas-fond de la vessie. Bull Soc Anat Paris 1827;2:199-205.

6.   Fenger C. Total extirpation of the vagina for carcinoma. Am J Obstet 1893;27:218-34.

7.   Herman G. A case of primary carcinoma of the vagina, leading to retention of urine. Lancet 1886;627-8.

8.   Roberts C. Trans Obstet Soc 1896;38:381.

9.   Lewers A. Trans Obstet Soc 1896;38:384.

10.   Wertheim E. Abdominale totalextirpation der vagina. Gynäk 1900;24:1393-6.

11.   Dougal D. Primary carcinoma of the vagina treated by hysterovaginectomy. Journal of Obstetrics and Gynaecology of the British Empire 1923;30:38-9.

12.   Holland E. A case of primary carcinoma of the vagina. Journal of Obstetrics and Gynaecology of the British Empire 1923;30:40-1.

13.   Stevens T. Squamous epithelioma of the vagina. Journal of Obstetrics and Gynaecology of the British Empire 1923;30:42-3.

14.   Brunschwig A. Complete excision of pelvic viscera for advanced carcinoma: a one-stage abdomino-perineal operation with end colostomy and bilateral ureteral implantation into the colon above the colostomy. Cancer 1948;1:177-83.

15.   Taussig F. Primary carcinoma of the vulva, vagina and female urethra. Surg Gynecol Obstet 1935;60:477-8.

16.   Messelt O. Primary cancer of the vagina. Surg Gynecol Obstet 1952;95:51-8.

17.   Murphy W. Primary vaginal cancer: Irradiation, management and end-results. Radiology 1957;68 (2):157-68.

18.   Kahle W, Leonhardt H, Platzer W. Genital organs. In Color atlas and textbook of human anatomy. Internal organs. Leonhardt H. Stuttgart: Georg Thieme 1978 (2), 256-315.

19.   Fu Y. Anatomy and Histology of the vagina. In Pathology of the Uterine Cervix, Vagina and Vulva. Fu Y. Philadelphia: Saunders 2002 (21), 30-5.

20.   Moore K. The Perineum and the Pelvis. In Clinically Oriented Anatomy. Moore K. Baltimore: Williams & Wilkins 1983, 294-418.

21.   Kahle W, Leonhardt H, Platzer W. Spinal cord and spinal nerves. In Color atlas and textbook of human anatomy. Nervous system and sensory organs. Leonhardt H. Stuttgart: Georg Thieme 1978 (3), pp86-7.

22.   Plentl A, Friedman E. Lymphatic system of female genital tract. The morphologic basis of oncologic diagnosis and therapy. Philadelphia: W.B. Saunders; 1971.

23.   Forsberg JG. Cervicovaginal epithelium: its origin and development. Am J Obstet Gynecol 1973;115(7):1025-43.

24. Robboy SJ, Taguchi O, Cunha GR. Normal development of the human female reproductive tract and alterations resulting from experimental exposure to diethylstilbestrol. Hum Pathol 1982;13(3):190-8.

25. Soutter W, Lambert J. Malignant disease of the vulva and vagina. In Gynaecology. Shaw R, Soutter W, Stanton S. New York: Churchill Livingstone 1997, pp569-85.

26. Fu Y. Benign and Malignant Epithelial Neoplasms of the Vagina. In Pathology of the Uterine Cervix, Vagina and Vulva. Fu Y. Philadelphia: Saunders 2002 (21), 232-68.

27. Benedet JL, Murphy KJ, Fairey RN, Boyes DA. Primary invasive carcinoma of the vagina. Obstet Gynecol 1983;62:715-9.

28. Sillman FH, Fruchter RG, Chen YS, Camilien L, Sedlis A, McTigue E. Vaginal intraepithelial neoplasia: risk factors for persistence, recurrence, and invasion and its management. Am J Obstet Gynecol 1997;176:93-9.

29. Diakomanolis E, Stefanidis K, Rodolakis A, Haidopoulos D, Sindos M, Chatzipappas I et al. Vaginal intraepithelial neoplasia: report of 102 cases. Eur J Gynaecol Oncol 2002;23(5):457-9.

30. Aho M, Vesterinen E, Meyer B, Purola E, Paavonen J. Natural history of vaginal intraepithelial neoplasia. Cancer 1991;68(1):195-7.

31. Rome RM, England PG. Management of vaginal intraepithelial neoplasia: A series of 132 cases with long-term follow-up. International Journal of Gynecological Cancer 2000;10(5):382-90.

32. Goodman A, Zukerberg LR, Nikrui N, Scully RE. Vaginal adenosis and clear cell carcinoma after 5-fluorouracil treatment for condylomas. Cancer 1991;68(7):1628-32.

33. Sugase M, Matsukura T. Distinct manifestations of human papillomaviruses in the vagina. Int J Cancer 1997;72(3):412-5.

34. Yalcin OT, Rutherford TJ, Chambers SK, Chambers JT, Schwartz PE. Vaginal intraepithelial neoplasia: treatment by carbon dioxide laser and risk factors for failure. Eur J Obstet Gynecol Reprod Biol 2003;106(1):64-8.

35. Creasman WT, Phillips JL, Menck HR. The national cancer data base report on cancer of the vagina. Cancer 1998;83:1033-40.

36. Palmer J, Biback S. Primary carcinoma of the vagina. Am J Obstet Gynecol 1954;67:377-97.

37. Whelton J, Kottmeier L. Primary carcinoma of the vagina. Acta obst et gynec scandinav 1962;41(22):22-40.

38. Fu YS, Reagan JW, Richart RM, Townsend DE. Nuclear DNA and histologic studies of genital lesions in diethylstilbestrol-exposed progeny. II. Intraepithelial glandular abnormalities. Am J Clin Pathol 1979;72(4):515-20.

39. Naves AE, Monti JA, Chichoni E. Basal cell-like carcinoma in the upper third of the vagina. Am J Obstet Gynecol 1980;137(1):136-7.

40. Young S, Leon M, Talerman A, Teresi M, Emmadi R. Polymorphous low-grade adenocarcinoma of the vulva and vagina: a tumour resembling adenoid cystic carcinoma. Int J Surg Pathol 2003;11(1):43-9.

41. Okagaki T, Ishida T, Hilgers RD. A malignant tumour of the vagina resembling synovial sarcoma: a light and electron microscopic study. Cancer 1976;37(5):2306-20.

42. Bing Z, Levine L, Lucci JA, Hatch SS, Eltorky MA. Primary small cell neuroendocrine carcinoma of the vagina: a clinicopathologic study. Arch Pathol Lab Med 2004;128(8):857-62.

43. Prasad CJ, Ray JA, Kessler S. Primary small cell carcinoma of the vagina arising in a background of atypical adenosis. Cancer 1992;70(10):2484-7.

44. Peters WA 3d, Kumar NB, Morley GW. Carcinoma of the vagina. Factors influencing treatment outcome. Cancer 1985;55:892-7.

45. Pride GL, Buchler DA. Carcinoma of vagina 10 or more years following pelvic irradiation therapy. Am J Obstet Gynecol 1977;127(5):513-7.

46. Kottmeier H. Annual report on the results of treatment in carcinoma of the uterus and vagina. International Federation of Gynecology and Obstetrics, Stockholm 1963;13.

47. Perez CA, Camel HM. Long-term follow-up in radiation therapy of carcinoma of the vagina. Cancer 1982;49(6):1308-15.

48. Prempree T, Viravathana T, Slawson RG, Wizenberg MJ, Cuccia CA. Radiation management of primary carcinoma of the vagina. Cancer 1977;40(1):109-18.

49. Brinton LA, Nasca PC, Mallin K, Schairer C, Rosenthal J, Rothenberg R et al. Case-control study of in situ and invasive carcinoma of the vagina. Gynecol Oncol 1990;38:49-54.

50. Daling JR, Madeleine MM, Schwartz SM, Shera KA, Carter JJ, McKnight B et al. A population-based study of squamous cell vaginal cancer: HPV and cofactors. Gynecol Oncol 2002;84(2):263-70.

51. Daling JR, Sherman KJ. Relationship between human papillomavirus infection and tumours of anogenital sites other than the cervix. IARC Sci Publ 1992;5:223-41.

52. Daling JR, Sherman KJ, Hislop TG, Maden C, Mandelson MT, Beckmann AM et al. Cigarette smoking and the risk of anogenital cancer. Am J Epidemiol 1992;135:180-9.

53. Hildesheim A, Han C-L, Brinton LA, Nasca PC, Richarts RM, Jones RB et al. Sexually transmitted agents and risk of carcinoma of the vagina. International Journal of Gynecological Cancer 1997;7:251-5.

54. Franco EL. Epidemiology of anogenital warts and cancer. Obstet Gynecol Clin North Am 1996;23:597-623.

55. Henson D, Tarone R. An epidemiologic study of cancer of the cervix, vagina, and vulva based on the third national cancer survey in the united states. Am J Obstet Gynecol 1977;129:525-32.

56. Peters RK, Mack TM, Bernstein L. Parallels in the epidemiology of selected anogenital carcinomas. J Natl Cancer Inst 1984;72:609-15.

57. Di Domenico A. Primary vaginal squamous cell carcinoma in the young patient. Gynecol Oncol 1989;35:181-7.

58. Pukkala E, Weiderpass E. Time trends in socio-economic differences in incidence rates of cancers of the breast and female genital organs (Finland, 1971-1995). Int J Cancer 1999;81(1):56-61.

59. Levi F, Randimbison L, La Vecchia C. Descriptive epidemiology of vulvar and vaginal cancers in Vaud, Switzerland, 1974-1994. Ann Oncol 1998;9(11):1229-32.

60. Sigvardsson S, Hardell L, Przybeck TR, Cloninger R. Increased cancer risk among Swedish female alcoholics. Epidemiology 1996;7(2):140-3.

61. Weiderpass E, Ye W, Tamimi R, Trichopolous D, Nyren O, Vainio H et al. Alcoholism and risk for cancer of the cervix uteri, vagina, and vulva. Cancer Epidemiol Biomarkers Prev 2001;10(8):899-901.

62. Luoto R, Kaprio J, Uutela A. Age at natural menopause and sociodemographic status in Finland. Am J Epidemiol 1994;139(1):64-76.

63. Available at http://www.molmine.com

64. Leminen A, Forss M, Lehtovirta P. Therapeutic and prognostic considerations in primary carcinoma of the vagina. Acta Obstet Gynecol Scand 1995;74:379-83.

65. Lee YC, Holcomb K, Buhl A, Holden J, Abulafia O. Rapid progression of primary vaginal squamous cell carcinoma in a young hiv-infected woman. Gynecol Oncol 2000;78:380-2.

66. Bjorge T, Dillner J, Anttila T, Engeland A, Hakulinen T, Jellum E et al. Prospective seroepidemiological study of role of human papillomavirus in non-cervical anogenital cancers. BMJ 1997;315:646-9.

67. Ikenberg H, Runge M, Goppinger A, Pfleiderer A. Human papillomavirus DNA in invasive carcinoma of the vagina. Obstet Gynecol 1990;76 (3 PT 1):432-8.

68. Ostrow RS, Manias DA, Clark BA, Fukushima M, Okagaki T, Twiggs LB et al. The analysis of carcinomas of the vagina for human papillomavirus DNA. Int J Gynecol Pathol 1988;7(4):308-14.

69. Marcus SL. Primary carcinoma of the vagina. Obstet Gynecol 1960;15:673-89.

70. Gallup DG, Talledo OE, Shah KJ, Hayes C. Invasive squamous cell carcinoma of the vagina: a 14-year study. Obstet Gynecol 1987;69:782-5.

71. Herbst AL, Green Jr TH, Ulfelder H. Primary carcinoma of the vagina. An analysis of 68 cases. Am J Obstet Gynecol 1970;106(2):210-8.

72. Choo YC, Anderson DG. Neoplasms of the vagina following cervical carcinoma. Gynecol Oncol 1982;14(1):125-32.

73. Rose PG, Herterick EE, Boutselis JG, Moeshberger M, Sachs L. Multiple primary gynecologic neoplasms. Am J Obstet Gynecol 1987;157:261-7.

74. Newman W, Cramer JD. The multicentric origin of carcinomas of the female anogenital tract. Surg Gynecol Obstet 1959;108:273-81.

75. Goodman A. Primary vaginal cancer. Surg Oncol Clin N Am 1998;7:347-61.

76. Frisch M, Melbye M. Risk of lung cancer in pre- and post-menopausal women with ano-genital malignancies. Int J Cancer 1995;62(5):508-11.

77. Sturgeon SR, Curtis RE, Johnson K, Ries L, Brinton LA. Second primary cancers after vulvar and vaginal cancers. Am J Obstet Gynecol 1996;174(3):929-33.

78. Franceschi S, Munoz N, Bosch XF, Snijders PJ, Walboomers JM. Human papillomavirus and cancers of the upper aerodigestive tract: a review of epidemiological and experimental evidence. Cancer Epidemiol Biomarkers Prev 1996;5(7):567-75.

79. Shamanin V, zur Hausen H, Lavergne D, Proby CM, Leigh IM, Neumann C et al. Human papillomavirus infections in nonmelanoma skin cancers from renal transplant recipients and nonimmunosuppressed patients. J Natl Cancer Inst 1996;88(12):802-11.

80. Bell J, Sevin BU, Averette H, Nadji M. Vaginal cancer after hysterectomy for benign disease: value of cytologic screening. Obstet Gynecol 1984;64:699-702.

81. Stuart GC, Allen HH, Anderson RJ. Squamous cell carcinoma of the vagina following hysterectomy. Am J Obstet Gynecol 1981;139(3):311-5.

82. Herman JM, Homesley HD, Dignan MB. Is hysterectomy a risk factor for vaginal cancer? JAMA 1986;256:601-3.

83. Chyle V, Zagars GK, Wheeler JA, Wharton JT, Delclos L. Definitive radiotherapy for carcinoma of the vagina: outcome and prognostic factors. Int J Radiat Oncol Biol Phys 1996;35:891-905.

84. Dixit S, Singhal S, Baboo HA. Squamous cell carcinoma of the vagina: a review of 70 cases. Gynecol Oncol 1993;48:80-7.

85. Foroudi F, Bull CA, Gebski V. Primary invasive cancer of the vagina: outcome and complications of therapy. Australas Radiol 1999;43:472-5.

86. Manetta A, Gutrecht EL, Berman ML, DiSaia PJ. Primary invasive carcinoma of the vagina. Obstet Gynecol 1990;76:639-42.

87. Stock RG, Chen AS, Seski J. A 30-year experience in the management of primary carcinoma of the vagina: analysis of prognostic factors and treatment modalities. Gynecol Oncol 1995;56:45-52.

88. Stock RG, Mychalczak B, Armstrong JG, Curtin JP, Harrison LB. The importance of brachytherapy technique in the management of primary carcinoma of the vagina. Int J Radiat Oncol Biol Phys 1992;24:747-53.

89. Urbanski K, Kojs Z, Reinfuss M, Fabisiak W. Primary invasive vaginal carcinoma treated with radiotherapy: analysis of prognostic factors. Gynecol Oncol 1996;60:16-21.

90. Merino MJ. Vaginal cancer: the role of infectious and environmental factors. Review 37 refs. Am J Obstet Gynecol 1991;165:1255-62.

91. Murad TM, Durant JR, Maddox WA, Dowling EA. The pathologic behavior of primary vaginal carcinoma and its relationship to cervical cancer. Cancer 1975;35:787-94.

92. Kapp DS, Fischer D, Grady KJ, Schwartz PE. Subsequent malignancies associated with carcinoma of the uterine cervix: including an analysis of the effect of patient and treatment parameters on incidence and sites of metachronous malignancies. Int J Radiat Oncol Biol Phys 1982;8(2):197-205.

93. Barrie j, Brunschwig A. Late second cancers of the cervix after apparent successful initial radiation therapy. American Journal of Roentgenology and Therapeutic Nuclear Medicine 1970;109:109-12.

94. Al-Kurdi M, Monaghan JM. Thirty-two years experience in management of primary tumours of the vagina. Br J Obstet Gynaecol 1981;88:1145-50.

95. Lindeque BG. The role of surgery in the management of carcinoma of the vagina. Review 33 refs. Baillieres Clin Obstet Gynaecol 1987;1:319-29.

96. Schraub S, Alauzet E, Schaffer P, Robillard J, Menegoz F. Epidemiologie descriptive des cancers gynecologiques et mammaires. Rev Fr Gynecol Obstet 1992;87:577-85.

97. Piura B, Rabinovich A, Cohen Y, Glezerman M. Primary squamous cell carcinoma of the vagina: report of four cases and review of the literature. Eur J Gynaecol Oncol 1998;19:60-3.

98. Moodley M, Moodley J, Chetty R, Herrington CS. The role of steroid contraceptive hormones in the pathogenesis of invasive cervical cancer: a review. International Journal of Gynecological Cancer 2003;13(2):103-10.

99. von Knebel Doeberitz M, Spitkovsky D, Ridder R. Interactions between steroid hormones and viral oncogenes in the pathogenesis of cervical cancer. Verh Dtsch Ges Pathol 1997;81:233-9.

100. Wharton JT, Tortolero-Luna G, Linares AC, Malpica A, Baker VV, Cook E et al. Vaginal intraepithelial neoplasia and vaginal cancer. Obstet Gynecol Clin North Am 1996;23:325-45.

101. Arbeit JM, Howley PM, Hanahan D. Chronic estrogen-induced cervical and vaginal squamous carcinogenesis in human papillomavirus type 16 transgenic mice. Proc Natl Acad Sci U S A 1996;93(7):2930-5.

102. Kim CJ, Um SJ, Kim TY, Kim EJ, Park TC, Kim SJ et al. Regulation of cell growth and HPV genes by exogenous estrogen in cervical cancer cells. International Journal of Gynecological Cancer 2000;10(2):157-64.

103. Mitrani-Rosenbaum S, Tsvieli R, Tur-Kaspa R. Oestrogen stimulates differential transcription of human papillomavirus type 16 in SiHa cervical carcinoma cells. J Gen Virol 1989;70(PT 8):2227-32.

104. Tedeschi CA, Rubin M, Krumholz BA. Six cases of women with diethylstilbestrol in utero demonstrating long-term manifestations and current evaluation guidelines. J Low Genit Tract Dis 2005;9(1):11-8.

105. Herbst AL. Diethylstilbestrol exposure--1984. N Engl J Med 1984;311(22):1433-5.

106. Kirkbride P, Fyles A, Rawlings GA, Manchul L, Levin W, Murphy KJ et al. Carcinoma of the vagina--experience at the Princess Margaret Hospital (1974-1989). Gynecol Oncol 1995;56:435-43.

107. Kucera H, Vavra N. Radiation management of primary carcinoma of the vagina: clinical and histopathological variables associated with survival. Gynecol Oncol 1991;40:12-6.

108. Viikki M, Pukkala E, Hakama M. Risk of endometrial, ovarian, vulvar, and vaginal cancers after a positive cervical cytology followed by negative histology. Obstet Gynecol 1998;92:269-73.

109. Perez CA, Grigsby PW, Garipagaoglu M, Mutch DG, Lockett MA. Factors affecting long-term outcome of irradiation in carcinoma of the vagina. Int J Radiat Oncol Biol Phys 1999;44:37-45.

110. Davis KP, Stanhope CR, Garton GR, Atkinson EJ, O'Brien PC. Invasive vaginal carcinoma: analysis of early-stage disease. Gynecol Oncol 1991;42:131-6.

111. Leung S, Sexton M. Radical radiation therapy for carcinoma of the vagina--impact of treatment modalities on outcome: Peter maccallum cancer institute experience 1970-1990. Int J Radiat Oncol Biol Phys 1993;25:413-8.

112. Yeh AM, Marcus Jr RB, Amdur RJ, Morgan LS, Million RR. Patterns of failure in squamous cell carcinoma of the vagina treated with definitive radiotherapy alone: what is the appropriate treatment volume? Int J Cancer 2001;96 Suppl:109-16.

113. Mock U, Kucera H, Fellner C, Knocke TH, Potter R. High-dose-rate (HDR) brachytherapy with or without external beam radiotherapy in the treatment of primary vaginal carcinoma: long-term results and side effects. Int J Radiat Oncol Biol Phys 2003;56(4):950-7.

114. Frank SJ, Jhingran A, Levenback C, Eifel PJ. Definitive radiation therapy for squamous cell carcinoma of the vagina. Int J Radiat Oncol Biol Phys 2005;62(1):138-47.

115. Nag S, Martinez-Monge R, Selman AE, Copeland LJ. Interstitial brachytherapy in the management of primary carcinoma of the cervix and vagina. Gynecol Oncol 1998;70(1):27-32.

116. Stryker JA. Radiotherapy for vaginal carcinoma: a 23-year review. Br J Radiol 2000;73(875):1200-5.

117. Tewari KS, DiSaia PJ. Radiation therapy for gynecologic cancer. J Obstet Gynaecol Res 2002;28(3):123-40.

118. Grigsby PW. Vaginal cancer. Curr Treat Options Oncol 2002;3(2):125-30.

119. Dalrymple JL, Russell AH, Lee SW, Scudder SA, Leiserowitz GS, Kinney WK et al. Chemoradiation for primary invasive squamous carcinoma of the vagina. International Journal of Gynecological Cancer 2004;14(1):110-7.

120. Grigsby PW. Cervical cancer: combined modality therapy. Cancer J 2001;7 Suppl 1:47-50.

121. Concurrent chemoradiation for cervical cancer. National Cancer Institute Clinical Announcement, 1999.

122. Thigpen JT, Blessing JA, Homesley HD, Berek JS, Creasman WT. Phase II trial of cisplatin in advanced or recurrent cancer of the vagina: a Gynecologic Oncology Group Study. Gynecol Oncol 1986;23(1):101-4.

123. Zanetta G, Lissoni A, Gabriele A, Landoni F, Colombo A, Perego P et al. Intense neoadjuvant chemotherapy with cisplatin and epirubicin for advanced or bulky cervical and vaginal adenocarcinoma. Gynecol Oncol 1997;64(3):431-5.

124. Tjalma WA, Monaghan JM, de Barros Lopes A, Naik R, Nordin AJ, Weyler JJ. The role of surgery in invasive squamous carcinoma of the vagina. Gynecol Oncol 2001;81(3):360-5.

125. Available at http://www.matrixscience.com

126. Kosary CL. FIGO stage, histology, histologic grade, age and race as prognostic factors in determining survival for cancers of the female gynecological system: an analysis of 1973-87 SEER cases of cancers of the endometrium, cervix, ovary, vulva, and vagina. Semin Surg Oncol 1994;10(1):31-46.

127. Ptackova B. Primary carcinoma of the vagina. Neoplasma 1968;15(3):309-22.

128. Malmström H, Engqvist M. Primary invasive cancer of the vagina. International Journal of Gynecological Cancer 1997;7:205-12.

129. Pingley S, Shrivastava SK, Sarin R, Agarwal JP, Laskar S, Deshpande DD et al. Primary carcinoma of the vagina: Tata memorial hospital experience. Int J Radiat Oncol Biol Phys 2000;46:101-8.

130. Budd W. The pathology and causes of cancer. The Lancet 1842;:266-70.

131. Franks l. What is cancer? In Cellular and molecular biology of cancer. Franks l, Teich n. New York: Oxford University Press 1998

132. von Hansemann D. Malignant growths and normal reproductive tissues. Lancet 1904;:251.

133. Boveri T. Zur Frage der Entstehung maligner Tumouren. Jena: Verlag von Gustav Fischer 1914.

134. Murray J. The chromosome imbalance theory of carcinogenesis. The Lancet 1929;297-8.

135. Deelman H, Van Erp J. Z Krebsforsch 1926;24:86.

136. MacKenzie I, Rous P. The experimental disclosure of latent neoplastic changes in tarred skin. J Exp Med 1941;73:391-424.

137. Friedewald W, Rous P. Ibid 1944;80:101.

138. Berenblum I, Shubik P. The persistence of latent tumour cells induced in the mouse's skin by a single application of 9:10-dimethyl-1:2-benzanthracene. Br J Cancer 1949;3(3):384-6.

139. Armitage P, Doll R. A two-stage theory of carcinogenesis in relation to the age distribution of human cancer. Br J Cancer 1957;11(2):161-9.

140. Armitage P, Doll R. The age distribution of cancer and a multi-stage theory of carcinogenesis. Br J Cancer 1954;8(1):1-12.

141. Hanahan D, Weinberg RA. The hallmarks of cancer. Saibo 2000;100(1):57-70.

142. Hahn WC, Weinberg RA. Rules for making human tumour cells. N Engl J Med 2002;347(20):1593-603.

143. Tessema M, Lehmann U, Kreipe H. Cell cycle and no end. Virchows Arch 2004;444:313-23.

144. Sellers WR, Kaelin Jr WG. Role of the retinoblastoma protein in the pathogenesis of human cancer. J Clin Oncol 1997;15(11):3301-12.

145. Sherr CJ. D-type cyclins. Trends Biochem Sci 1995;20(5):187-90.

146. Lane DP. Cancer. p53, guardian of the genome. Nature 1992;358(6381):15-6.

147. Cadwell C, Zambetti GP. Regulators and mediators of the p53 tumour suppressor. J Cell Biochem Suppl 1998;30-31:43-9.

148. Agarwal ML, Agarwal A, Taylor WR, Stark GR. p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. Proc Natl Acad Sci U S A 1995;92(18):8493-7.

149. Lundberg AS, Weinberg RA. Control of the cell cycle and apoptosis. Eur J Cancer 1999;35(4):531-9.

150. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM et al. WAF1, a potential mediator of p53 tumour suppression. Saibo 1993;75(4):817-25.

151. Park MT, Lee SJ. Cell cycle and cancer. Journal of Biochemistry and Molecular Biology 2003;36(1):60-5.

152. Hahn WC, Weinberg RA. Modelling the molecular circuitry of cancer. Nat Rev Cancer 2002;2(5):331-41.

153. Cadwell C, Zambetti GP. The effects of wild-type p53 tumour suppressor activity and mutant p53 gain-of-function on cell growth. Gene 2001;277(1-2):15-30.

154. ter Harmsel B, van Belkum A, Quint W, Pronk A, Kuijpers J, Ramaekers F et al. p53 and human papilloma virus type 16 in cervical intraepithelial neoplasia and carcinoma. Int J Gynecol Pathol 1995;14(2):125-33.

155. Auer G, Steinbeck R, Zetterberg A. Molecular Markers in Diagnostic pathology. The computerized Cytology and Histology Laboratory 1994;129-42.

156. Fox H. Clinical value of new techniques of gynecological tumour assessment. International Journal of Gynecological Cancer 1997;7:337-49.

157. Milde-Langosch K, Riethdorf S. Role of cell-cycle regulatory proteins in gynecological cancer. J Cell Physiol 2003;196(2):224-44.

158. Zur Hausen H. Papillomavirus causing cancer: Evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 2000;92:690-8.

159. Koyamatsu Y, Yokoyama M, Nakao Y, Fukuda K, Saito T, Matsukuma K et al. A comparative analysis of human papillomavirus types 16 and 18 and expression of p53 gene and Ki-67 in cervical, vaginal, and vulvar carcinomas. Gynecol Oncol 2003;90(3):547-51.

160. Skomedal H, Kristensen G, Helland A, Nesland JM, Kooi S, Borresen AL et al. TP53 gene mutations and protein accumulation in primary vaginal carcinomas. Br J Cancer 1995;72(1):129-33.

161. Skomedal H, Kristensen G, Holm R. Expression of retinoblastoma tumour suppressor gene protein, epidermal growth factor receptor, and c-erbB-2 oncoprotein in primary vaginal carcinomas. Gynecol Oncol 1995;59(3):379-83.

162. Lerma E, Matias-Guiu X, Lee SJ, Prat J. Squamous cell carcinoma of the vulva: study of ploidy, HPV, p53, and pRb. Int J Gynecol Pathol 1999;18(3):191-7.

163. Kagie MJ, Kenter GG, Zomerdijk-Nooijen Y, Hermans J, Schuuring E, Timmers PJ et al. Human papillomavirus infection in squamous cell carcinoma of the vulva, in various synchronous epithelial changes and in normal vulvar skin. Gynecol Oncol 1997;67(2):178-83.

164. Kagie MJ, Kenter GG, Tollenaar RA, Hermans J, Trimbos JP, Fleuren GJ. p53 protein overexpression, a frequent observation in squamous cell carcinoma of the vulva and in various synchronous vulvar epithelia, has no value as a prognostic parameter. Int J Gynecol Pathol 1997;16(2):124-30.

165. Lee MH, Yang HY. Negative regulators of cyclin-dependent kinases and their roles in cancers. Cell Mol Life Sci 2001;58(12-13):1907-22.

54

166. van de Putte G, Holm R, Lie AK, Trope CG, Kristensen GB. Expression of p27, p21, and p16 protein in early squamous cervical cancer and its relation to prognosis. Gynecol Oncol 2003;89(1):140-7.

167. Knopp S, Bjorge T, Nesland JM, Trope C, Holm R. Cyclins D1, D3, E, and A in vulvar carcinoma patients. Gynecol Oncol 2005;97(3):733-9.

168. Shiohara S, Shiozawa T, Miyamoto T, Feng YZ, Kashima H, Kurai M et al. Expression of cyclins, p53, and Ki-67 in cervical squamous cell carcinomas: overexpression of cyclin A is a poor prognostic factor in stage Ib and II disease. Virchows Arch 2005;446(6):626-33.

169. Van de Putte G, Kristensen GB, Lie AK, Baekelandt M, Holm R. Cyclins and proliferation markers in early squamous cervical carcinoma. Gynecol Oncol 2004;92(1):40-6.

170. Cho NH, Kim YT, Kim JW. Correlation between G1 cyclins and HPV in the uterine cervix. Int J Gynecol Pathol 1997;16(4):339-47.

171. Keyomarsi K, Tucker SL, Buchholz TA, Callister M, Ding Y, Hortobagyi GN et al. Cyclin E and survival in patients with breast cancer. N Engl J Med 2002;347(20):1566-75.

172. Keating JT, Ince T, Crum CP. Surrogate biomarkers of HPV infection in cervical neoplasia screening and diagnosis. Adv Anat Pathol 2001;8(2):83-92.

173. Klaes R, Friedrich T, Spitkovsky D, Ridder R, Rudy W, Petry U et al. Overexpression of p16(INK4A) as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. Int J Cancer 2001;92(2):276-84.

174. Riethdorf L, Riethdorf S, Lee KR, Cviko A, Loning T, Crum CP. Human papillomaviruses, expression of p16, and early endocervical glandular neoplasia. Hum Pathol 2002;33(9):899-904.

175. Hale RJ, Buckley CH, Fox H, Williams J. Prognostic value of c-erbB-2 expression in uterine cervical carcinoma. J Clin Pathol 1992;45(7):594-6.

176. Mitra AB, Murty VV, Pratap M, Sodhani P, Chaganti RS. ERBB2 (HER2/neu) oncogene is frequently amplified in squamous cell carcinoma of the uterine cervix. Cancer Res 1994;54(3):637-9.

177. Zhang A, Maner S, Betz R, Angstrom T, Stendahl U, Bergman F et al. Genetic alterations in cervical carcinomas: frequent low-level amplifications of oncogenes are associated with human papillomavirus infection. Int J Cancer 2002;101(5):427-33.

178. Rosty C, Couturier J, Vincent-Salomon A, Genin P, Freneaux P, Sigal-Zafrani B et al. Overexpression/amplification of HER-2/neu is uncommon in invasive carcinoma of the uterine cervix. Int J Gynecol Pathol 2004;23(1):13-7.

179. Paul MK, Mukhopadhyay AK. Tyrosine kinase - Role and significance in Cancer. Int J Med Sci 2004;1(2):101-15.

180. Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. N Engl J Med 2005;353(2):172-87.

181. Alison MR. Assessing cellular proliferation: what's worth measuring? Hum Exp Toxicol 1995;14(12):935-44.

182. Auer G, Askensten U, Ahrens O. Cytophotometry. Hum Pathol 1989;20(6):518-27.

183. Gerlach C, Golding M, Larue L, Alison MR, Gerdes J. Ki-67 immunoexpression is a robust marker of proliferative cells in the rat. Lab Invest 1997;77(6):697-8.

184. Oka K, Arai T. MIB1 growth fraction is not related to prognosis in cervical squamous cell carcinoma treated with radiotherapy. Int J Gynecol Pathol 1996;15(1):23-7.

185. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182(3):311-22.

186. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 1983;31(1):13-20.

187. Cattoretti G, Becker MH, Key G, Duchrow M, Schluter C, Galle J et al. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 1992;168(4):357-63.

188. Wang J, Chenivesse X, Henglein B, Brechot C. Hepatitis B virus integration in a cyclin A gene in a hepatocellular carcinoma. Nature 1990;343(6258):555-7.

189. Yam CH, Fung TK, Poon RY. Cyclin A in cell cycle control and cancer. Cell Mol Life Sci 2002;59(8):1317-26.

190. Basset P, Okada A, Chenard MP, Kannan R, Stoll I, Anglard P et al. Matrix metalloproteinases as stromal effectors of human carcinoma progression: therapeutic implications. Matrix Biol 1997;15(8-9):535-41.

191. Reich R, Blumenthal M, Liscovitch M. Role of phospholipase D in laminin-induced production of gelatinase A (MMP-2) in metastatic cells. Clin Exp Metastasis 1995;13(2):134-40.

192. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumourigenesis. Saibo 1996;86(3):353-64.

193. Kranenburg O, Gebbink MF, Voest EE. Stimulation of angiogenesis by Ras proteins. Biochim Biophys Acta 2004;1654(1):23-37.

194. Giannelli G, Antonaci S. Biological and clinical relevance of Laminin-5 in cancer. Clin Exp Metastasis 2000;18(6):439-43.

195. Patarroyo M, Tryggvason K, Virtanen I. Laminin isoforms in tumour invasion, angiogenesis and metastasis. Semin Cancer Biol 2002;12(3):197-207.

196. Burgeson RE, Chiquet M, Deutzmann R, Ekblom P, Engel J, Kleinman H et al. A new nomenclature for the laminins. Matrix 1994;14:209-11.

197. Tryggvason K. The laminin family. Curr Opin Cell Biol 1993;5:877-82.

198. Katayama M, Sanzen N, Funakoshi A, Sekiguchi K. Laminin gamma2-chain fragment in the circulation: a prognostic indicator of epithelial tumour invasion. Cancer Res 2003;63(1):222-9.

199. Miyazaki K, Kikkawa Y, Nakamura A, Yasumitsu H, Umeda M. A large cell-adhesive scatter factor secreted by human gastric carcinoma cells. Proc Natl Acad Sci U S A 1993;90:11767-71.

200. Ryan MC, Tizard R, VanDevanter DR, Carter WG. Cloning of the lama3 gene encoding the alpha 3 chain of the adhesive ligand epiligrin. Expression in wound repair. J Biol Chem 1994;269:22779-87.

201. Vailly J, Szepetowski P, Mattei MG, Pedeutour F, Burgeson R, Ortonne JP et al. The genes for nicein/kalinin 125- and 100-kDa subunits, candidates for junctional epidermolysis bullosa, map to chromosomes 1q32 and 1q25-q31. Genomics 1994;21(1):286-8.

202. Aumailley M, El Khal A, Knoss N, Tunggal L. Laminin 5 processing and its integration into the ECM. Matrix Biol 2003;22(1):49-54.

203. Larjava H, Salo T, Haapasalmi K, Kramer RH, Heino J. Expression of integrins and basement membrane components by wound keratinocytes. J Clin Invest 1993;92:1425-35.

204. Pyke C, Romer J, Kallunki P, Lund LR, Ralfkiaer E, Dano K et al. The gamma 2 chain of kalinin/laminin 5 is preferentially expressed in invading malignant cells in human cancers. Am J Pathol 1994;145:782-91.

205. Goldfinger LE, Stack MS, Jones JC. Processing of laminin-5 and its functional consequences: role of plasmin and tissue-type plasminogen activator. J Cell Biol 1998;141(1):255-65.

206. Salo S, Haakana H, Kontusaari S, Hujanen E, Kallunki T, Tryggvason K. Laminin-5 promotes adhesion and migration of epithelial cells: identification of a migration-related element in the gamma2 chain gene (lamc2) with activity in transgenic mice in process citation. Matrix Biol 1999;18:197-210.

207. Koshikawa N, Minegishi T, Sharabi A, Quaranta V, Seiki M. Membrane-type matrix metalloproteinase-1 (MT1-MMP) is a processing enzyme for human laminin gamma 2 chain. J Biol Chem 2005;280(1):88-93.

208. Kainulainen T, Autio-Harmainen H, Oikarinen A, Salo S, Tryggvason K, Salo T. Altered distribution and synthesis of laminin-5 (kalinin) in oral lichen planus, epithelial dysplasias and squamous cell carcinomas. Br J Dermatol 1997;136:331-6.

209. Lundgren C, Frankendal B, Silfverswärd C, Nilsson B, Tryggvason K, Auer G et al. Laminin-5 gamma2-chain expression and DNA ploidy as predictors of prognosis in endometrial carcinoma. Med Oncol 2003;20(2):147-56.

210. Orian-Rousseau V, Aberdam D, Rousselle P, Messent A, Gavrilovic J, Meneguzzi G et al. Human colonic cancer cells synthesize and adhere to laminin-5. Their adhesion to laminin-5 involves multiple receptors among which is integrin alpha2beta1. J Cell Sci 1998;111:1993-2004.

211. Soini Y, Maatta M, Salo S, Tryggvason K, Autio-Harmainen H. Expression of the laminin gamma 2 chain in pancreatic adenocarcinoma. J Pathol 1996;180:290-4.

212. Sordat I, Bosman FT, Dorta G, Rousselle P, Aberdam D, Blum AL et al. Differential expression of laminin-5 subunits and integrin receptors in human colorectal neoplasia. J Pathol 1998;185:44-52.

213. Tani T, Karttunen T, Kiviluoto T, Kivilaakso E, Burgeson RE, Sipponen P et al. Alpha 6 beta 4 integrin and newly deposited laminin-1 and laminin-5 form the adhesion mechanism of gastric carcinoma. Continuous expression of laminins but not that of collagen vii is preserved in invasive parts of the carcinomas: implications for ac. Am J Pathol 1996;149:781-93.

214. Ono Y, Nakanishi Y, Ino Y, Niki T, Yamada T, Yoshimura K et al. Clinocopathologic significance of laminin-5 gamma2 chain expression in squamous cell carcinoma of the tongue: immunohistochemical analysis of 67 lesions. Cancer 1999;85:2315-21.

215. Lenander C, Habermann JK, Ost A, Nilsson B, Schimmelpenning H, Tryggvason K et al. Laminin-5 gamma 2 chain expression correlates with unfavorable prognosis in colon carcinomas. Anal Cell Pathol 2001;22(4):201-9.

216. Hao J, Jackson L, Calaluce R, McDaniel K, Dalkin BL, Nagle RB. Investigation into the mechanism of the loss of laminin 5 (alpha3beta3gamma2) expression in prostate cancer. Am J Pathol 2001;158(3):1129-35.

217. Martin KJ, Kwan CP, Nagasaki K, Zhang X, O'Hare MJ, Kaelin CM et al. Down-regulation of laminin-5 in breast carcinoma cells. Mol Med 1998;4(9):602-13.

218. Skyldberg B, Salo S, Eriksson E, Aspenblad U, Moberger B, Tryggvason K et al. Laminin-5 as a marker of invasiveness in cervical lesions. J Natl Cancer Inst 1999;91:1882-7.

219. Nordstrom B, Einhorn N, Silfverswärd C, Sjovall K, Tryggvason K, Auer G. Laminin-5 gamma 2 chain as an invasivity marker for uni- and multifocal lesions in the lower anogenital tract. International Journal of Gynecological Cancer 2002;12(1):105-9.

220. Katayama M, Sekiguchi K. Laminin-5 in epithelial tumour invasion. J Mol Histol 2004;35(3):277-86.

221. Katayama M, Funakoshi A, Sumii T, Sanzen N, Sekiguchi K. Laminin gamma2-chain fragment circulating level increases in patients with metastatic pancreatic ductal cell adenocarcinomas. Cancer Lett 2005;225(1):167-76.

222. Longo LD. Classic pages in obstetrics and gynecology. The chromosome number in man. Joe Hin Tjio and Albert Levan. Hereditas, vol. 42, pp. 1-6, 1956. Am J Obstet Gynecol 1978;130(6):722.

223. Venter JC, Adams MD, Myers EW, Li PW, Mural RJ, Sutton GG et al. The sequence of the human genome. Science 2001;291(5507):1304-51.

224. Baak JP, Path FR, Hermsen MA, Meijer G, Schmidt J, Janssen EA. Genomics and proteomics in cancer. Eur J Cancer 2003;39(9):1199-215.

225. Caspersson TO. History of the development of cytophotometry from 1935 to the present. Anal Quant Cytol Histol 1987;9(1):2-6.

226. Feulgen R, Rossenbeck H. Mikroskopisch-chemischer Nachweis einer Nukleinsaure vom Typus der Thymonukleinsaure und die darauf beruhende elektivd 'Farbung van Zellkernen in mikroskonischen Pranaraten. Phvsiol Chem 1924;135(203).

227. Bauer TW, Tubbs RR, Edinger MG, Suit PF, Gephardt GN, Levin HS. A prospective comparison of DNA quantitation by image and flow cytometry. Am J Clin Pathol 1990;93(3):322-6.

228. Kaern J, Wetteland J, Trope CG, Farrants GW, Juhng SW, Pettersen EO et al. Comparison between flow cytometry and image cytometry in ploidy distribution assessments in gynecologic cancer. Cytometry 1992;13(3):314-21.

229. Lundgren C, Auer G, Frankendal B, Moberger B, Nilsson B, Nordstrom B. Nuclear DNA content, proliferative activity, and p53 expression related to clinical and histopathologic features in endometrial carcinoma. International Journal of Gynecological Cancer 2002;12(1):110-8.

230. Forsslund G, Esposti PL, Nilsson B, Zetterberg A. The prognostic significance of nuclear DNA content in prostatic carcinoma. Cancer 1992;69(6):1432-9.

231. Strang P, Stendahl U, Bergstrom R, Frankendal B, Tribukait B. Prognostic flow cytometric information in cervical squamous cell carcinoma: a multivariate analysis of 307 patients. Gynecol Oncol 1991;43(1):3-8.

232. Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F et al. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumours. Science 1992;258(5083):818-21.

233. Forozan F, Karhu R, Kononen J, Kallioniemi A, Kallioniemi OP. Genome screening by comparative genomic hybridization. Trends Genet 1997;13(10):405-9.

234. Knuutila S. Cytogenetics and molecular pathology in cancer diagnostics. Ann Med 2004;36(3):162-71.

235. Ried T, Heselmeyer-Haddad K, Blegen H, Schrock E, Auer G. Genomic changes defining the genesis, progression, and malignancy potential in solid human tumours: a phenotype/genotype correlation. Genes Chromosomes Cancer 1999;25(3):195-204.

236. Auer GU, Heselmeyer KM, Steinbeck RG, Munck-Wikland E, Zetterberg AD. The relationship between aneuploidy and p53 overexpression during genesis of colorectal adenocarcinoma. Virchows Arch 1994;424(4):343-7.

237. Heselmeyer K, Schrock E, du Manoir S, Blegen H, Shah K, Steinbeck R et al. Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix. Proc Natl Acad Sci U S A 1996;93(1):479-84.

238. Heselmeyer K, Macville M, Schrock E, Blegen H, Hellström AC, Shah K et al. Advanced-stage cervical carcinomas are defined by a recurrent pattern of chromosomal aberrations revealing high genetic instability and a consistent gain of chromosome arm 3q. Genes Chromosomes Cancer 1997;19:233-40.

239. Heselmeyer K, Hellström AC, Blegen H, Schrock E, Silfverswärd C, Shah K et al. Primary carcinoma of the fallopian tube: comparative genomic hybridization reveals high genetic instability and a specific, recurring pattern of chromosomal aberrations. Int J Gynecol Pathol 1998;17:245-54.

240. Jee KJ. Loss in 3p and 4p and gain of 3q are concomitant aberrations in squamous cell carcinoma of the vulva. Mod Pathol 2001;14(5):377-81.

241. Stoltzfus P, Heselmeyer-Haddad K, Castro J, White N, Silfverswärd C, Sjovall K et al. Gain of chromosome 3q is an early and consistent genetic aberration in carcinomas of the vulva. International Journal of Gynecological Cancer 2005;15(1):120-6.

242. Heselmeyer-Haddad K, Sommerfeld K, White NM, Chaudhri N, Morrison LE, Palanisamy N et al. Genomic amplification of the human telomerase gene (TERC) in pap smears predicts the development of cervical cancer. Am J Pathol 2005;166(4):1229-38.

243. Masutomi K, Yu EY, Khurts S, Ben-Porath I, Currier JL, Metz GB et al. Telomerase maintains telomere structure in normal human cells. Saibo 2003;114(2):241-53.

244. Hahn WC. Role of telomeres and telomerase in the pathogenesis of human cancer. J Clin Oncol 2003;21(10):2034-43.

245. Wilkins MR, Sanchez JC, Gooley AA, Appel RD, Humphery-Smith I, Hochstrasser DF et al. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Eng Rev 1996;13:19-50.

246. O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. J Biol Chem 1975;250(10):4007-21.

247. Misek DE, Imafuku Y, Hanash SM. Application of proteomic technologies to tumour analysis. Pharmacogenomics 2004;5(8):1129-37.

248. Wu W, Hu W, Kavanagh JJ. Proteomics in cancer research. International Journal of Gynecological Cancer 2002;12(5):409-23.

249. Appel RD, Vargas JR, Palagi PM, Walther D, Hochstrasser DF. Melanie II--a third-generation software package for analysis of two-dimensional electrophoresis images: II. Algorithms. Electrophoresis 1997;18(15):2735-48.

250. Rosengren AT, Salmi JM, Aittokallio T, Westerholm J, Lahesmaa R, Nyman TA et al. Comparison of PDQuest and Progenesis software packages in the analysis of two-dimensional electrophoresis gels. Proteomics 2003;3(10):1936-46.

251. Roblick UJ, Hirschberg D, Habermann JK, Palmberg C, Becker S, Kruger S et al. Sequential proteome alterations during genesis and progression of colon cancer. Cell Mol Life Sci 2004;61(10):1246-55.

252. Alaiya AA, Roblick UJ, Franzen B, Bruch HP, Auer G. Protein expression profiling in human lung, breast, bladder, renal, colorectal and ovarian cancers. J Chromatogr B Analyt Technol Biomed Life Sci 2003;787(1):207-22.

253. Franzen B, Linder S, Alaiya AA, Eriksson E, Fujioka K, Bergman AC et al. Analysis of polypeptide expression in benign and malignant human breast lesions. Electrophoresis 1997;18(3-4):582-7.

254. Alaiya AA, Franzen B, Hagman A, Silfverswärd C, Moberger B, Linder S et al. Classification of human ovarian tumours using multivariate data analysis of polypeptide expression patterns. Int J Cancer 2000;86(5):731-6.

255. Bae SM, Lee CH, Cho YL, Nam KH, Kim YW, Kim CK et al. Two-dimensional gel analysis of protein expression profile in squamous cervical cancer patients. Gynecol Oncol 2005;99(1):26-35.

256. Perez CA, Camel HM, Galakatos AE, Grigsby PW, Kuske RR, Buchsbaum G et al. Definitive irradiation in carcinoma of the vagina: long-term evaluation of results. Int J Radiat Oncol Biol Phys 1988;15:1283-90.

257. Habermann J, Lenander C, Roblick UJ, Kruger S, Ludwig D, Alaiya A et al. Ulcerative colitis and colorectal carcinoma: DNA-profile, laminin-5 gamma2 chain and cyclin A expression as early markers for risk assessment. Scand J Gastroenterol 2001;36(7):751-8.

258. Auer GU, Caspersson TO, Wallgren AS. DNA content and survival in mammary carcinoma. Anal Quant Cytol 1980;2(3):161-5.

259. Schimmelpenning H, Falkmer UG, Hamper K, Seifert G, Auer GU. Variations in Feulgen stainability of epithelial parenchymal cells extracted from paraffin-embedded salivary gland specimens. Cytometry 1990;11(4):475-80.

260. Steinbeck RG, Auer GU, Zetterberg AD. Reliability and significance of DNA measurements in interphase nuclei and division figures in histological sections. Eur J Cancer 1999;35(5):787-95.

261. Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlee F, Hildesheim A et al. Improved amplification of genital human papillomaviruses. J Clin Microbiol 2000;38(1):357-61.

262. Franzen B, Iwabuchi H, Kato H, Lindholm J, Auer G. Two-dimensional polyacrylamide gel electrophoresis of human lung cancer: qualitative aspects of tissue preparation in relation to histopathology. Electrophoresis 1991;12(7-8):509-15.

263. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.

264. Okuzawa K, Franzen B, Lindholm J, Linder S, Hirano T, Bergman T et al. Characterization of gene expression in clinical lung cancer materials by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis 1994;15(3-4):382-90.

265. Franzen B, Linder S, Okuzawa K, Kato H, Auer G. Nonenzymatic extraction of cells from clinical tumour material for analysis of gene expression by two-dimensional polyacrylamide gel electrophoresis. Electrophoresis 1993;14(10):1045-53.

266. Rabilloud T, Vuillard L, Gilly C, Lawrence JJ. Silver-staining of proteins in polyacrylamide gels: a general overview. Cell Mol Biol (Noisy-le-grand) 1994;40(1):57-75.

267. Alaiya AA, Franzen B, Hagman A, Dysvik B, Roblick UJ, Becker S et al. Molecular classification of borderline ovarian tumours using hierarchical cluster analysis of protein expression profiles. Int J Cancer 2002;98(6):895-9.

268. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 1998;95(25):14863-8.

269. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 1999;286(5439):531-7.

270. Fellenberg K, Hauser NC, Brors B, Neutzner A, Hoheisel JD, Vingron M. Correspondence analysis applied to microarray data. Proc Natl Acad Sci U S A 2001;98(19):10781-6.

271. Enterline PE. Pitfalls in epidemiological research. An examination of the asbestos literature. J Occup Med 1976;18(3):150-6.

272. Pettersson B. Menstruation span--a time-limited risk factor for endometrial carcinoma. Acta Obstet Gynecol Scand 1986;65(3):247-55.

273. Hoffman MS, DeCesare SL, Roberts WS, Fiorica JV, Finan MA, Cavanagh D. Upper vaginectomy for in situ and occult, superficially invasive carcinoma of the vagina. Am J Obstet Gynecol 1992;166(1 Pt 1):30-3.

274. Statistiska Centralbyrån SS. Demografiska rapporter, http://www.scb.se 2002.

275. Statistiska Centralbyrån SS. ULF http://www.scb.se.

276. Statistiska Centralbyrån SS. Befolkningsstatistik, del3, http://www.scb.se 2001.

277. Canavan TP, Cohen D. Vulvar cancer. Am Fam Physician 2002;66(7):1269-74.

278. Andersson S, Rylander E, Larsson B, Strand A, Silfverswärd C, Wilander E. The role of human papillomavirus in cervical adenocarcinoma carcinogenesis. Eur J Cancer 2001;37(2):246-50.

279. Houghton CR, Iversen T. Squamous cell carcinoma of the vagina: a clinical study of the location of the tumour. Gynecol Oncol 1982;13:365-72.

280. Eddy GL, Marks Jr RD, Miller MC 3d, Underwood Jr PB. Primary invasive vaginal carcinoma. Am J Obstet Gynecol 1991;165:292-6.

281. Malmström H, Simonsen E, Trope C. Primary invasive squamous cell carcinoma of the vagina. Acta Obstet Gynecol Scand 1989;68:411-5.

282. Kirchhoff M, Rose H, Petersen BL, Maahr J, Gerdes T, Lundsteen C et al. Comparative genomic hybridization reveals a recurrent pattern of chromosomal aberrations in severe dysplasia/carcinoma in situ of the cervix and in advanced-stage cervical carcinoma. Genes Chromosomes Cancer 1999;24(2):144-50.

283. Cavalcanti SM, Deus FC, Zardo LG, Frugulhetti IC, Oliveira LH. Human papillomavirus infection and cervical cancer in Brazil: a retrospective study. Mem Inst Oswaldo Cruz 1996;91(4):433-40.

284. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J Pathol 1999;189(1):12-9.

285. Kiechle M, Jacobsen A, Schwarz-Boeger U, Hedderich J, Pfisterer J, Arnold N. Comparative genomic hybridization detects genetic imbalances in primary ovarian carcinomas as correlated with grade of differentiation. Cancer 2001;91(3):534-40.

286. Suehiro Y. Genetic aberrations detected by comparative genomic hybridization predict outcome in patients with endometrioid carcinoma. Genes Chromosomes Cancer 2000;29(1):75-82.

287. Dwek MV, Alaiya AA. Proteome analysis enables separate clustering of normal breast, benign breast and breast cancer tissues. Br J Cancer 2002;22(2):305-7.