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Prognostic factors for squamous cell cervical cancer

Tumor markers, hormones, smoking, and S-phase fraction

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In loving memory of my parents Ingegerd and Carl-Erik Berg
and to my family Dag, Björn, Vidar and Frej

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ABSTRACT

Cervical cancer is the second most common malignancy in women worldwide and one of the leading causes of cancer mortality globally. In patients with invasive cervical cancer prognostic factors are of value for the choice of treatment, monitoring of treatment and follow-up. The most important clinical prognostic factors are stage, tumor volume, parametrial infiltration, vascular invasion, lymph node metastases, and distant metastases. An improved estimation of the prognosis of cervical cancer is desirable, especially in early cancer stages.

The aim of this research was to study possible associations between tumor markers, female sex steroids, smoking, S-phase fraction (SPF), and prognosis in invasive squamous cell cervical cancer (SCC). The study comprised 190 patients with SCC, stages IB-IV, admitted to the Department of Gynecologic Oncology at Norrland University Hospital in Umeå between September 1984 and October 1990. Ten year mortality was estimated.

In study I, of a total of 103 patients, it was found that increased tumor growth, measured by the DNA SPF, was associated with elevated serum progesterone and smoking in the premenopausal patients and with aneuploidy in the whole group.

In study II, comprising 128 patients, survival length related to hormone levels and SPF was evaluated in women who died of cervical cancer. In both pre- and postmenopausal women, who died of cervical cancer, SPF at or above 12% was correlated with reduced survival. There was significant positive correlation between a low serum estradiol/progesterone ratio and short survival in those premenopausal women who died of cancer ($p=0.02$).

In study III, ten-year follow-up results in 128 women were compared with the expression of ten relevant tumor markers, assessed by immunohistochemistry. The overall ten-year survival rate in patients with low COX-2 and high CD4+ expression was 76%, versus 53% in the remaining women. The survival rate with absent p53 and high COX-2 expression in the tumors was 42%, versus 71%, while the corresponding figure for the combination of high COX-2 intensity and expression of c-myc was 27%, versus 62%. None of the single markers correlated significantly with the outcome in the final Cox regression analyses, while five combinations did.

Study IV addressed possible associations between selected tumor markers and cofactors in SCC. Ten tumor markers were examined in 128 patients. Smoking habits and previous oral contraceptive use were recorded. Serum estradiol and progesterone levels were evaluated in 80 women. Highly significant associations were found between strong c-myc staining and increased progesterone, low EGFR staining and high serum estradiol, and absence of p53 staining and smoking. There was an association between absence of p53 and high serum progesterone.

In study V, LRIG1 expression was studied in 128 patients and was compared with expression of nine other tumor markers, smoking history, hormone levels, and prognosis. LRIG1 appears to be a significant prognostic predictor in early stage SCC, independent of the other tumor markers that were studied. Diminished expression in advanced cancer stages and the inverse correlation to serum progesterone and smoking indicate that LRIG1 is a tumor suppressor in squamous cell cervical cancer.

Conclusion: The results of these studies support a role of progesterone as a promoter of cervical cancer and indicate that smoking is associated with tumor progression. A combination of tumor markers might be of help in prognostic prediction. LRIG1 acts as a tumor suppressor. These findings might contribute towards greater understanding of prognostic prediction of squamous cell cervical cancer.

Key words: Cervical cancer, squamous cell carcinoma, sex steroid hormones, smoking, S-phase fraction, tumor markers, LRIG1, prognosis

PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Correlations between serum progesterone and smoking, and the growth fraction of cervical squamous cell carcinoma.
Lindström A, Backstrom T, Hellberg D, Tribukait B, Strang P, Stendahl U.
Anticancer Res 2000; 12:149-57.

II. Correlation between serum estradiol/progesterone ratio and survival length in invasive squamous cell cervical cancer.
Hellberg D, **Lindström AK**, Stendahl U.
Anticancer Res 2005; 25:611-16

III. Predicting the outcome of squamous cell carcinoma of the uterine cervix using combinations of individual tumor marker expressions.
Lindström, AK, Stendahl U, Tot T, Lidström BM, Hellberg D.
Anticancer Res 2007; 27:1609-16

IV. Associations between ten biological tumor markers in squamous cell cervical cancer and serum estradiol, serum progesterone and smoking.
Lindström AK, Stendahl U, Tot T, Hellberg D.
Anticancer Res. 2007; 27:1401-16

V. LRIG1 and squamous epithelial uterine cervical cancer: correlation to prognosis, other tumor markers, sex steroid hormones and smoking.
Lindström AK, Ekman K, Stendahl U, Tot T, Henriksson R, Hedman H,Hellberg D.
Int J Gynecol Cancer. 2008; 18:312-7.

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Other relevant papers by the author:

Diagnostic, endocrinological, behavioral, and DNA ploidy differences between squamous cell and adenomatous carcinoma of the cervix uteri.
Lindström AK, Hellberg D, Bäckström T, Stendahl U.
Oncol Res. 2004;14(7-8):321-4.

Discrepancies in expression and prognostic value of tumor markers in adenocarcinoma and squamous cell carcinoma in cervical cancer.
Lindström AK, Tot T, Stendahl U, Syrjänen S, Syrjänen K, Hellberg D.
Anticancer Res. 2009 Jul;29(7):2577-8.

LRIG2 in contrast to LRIG1 predicts poor survival in early-stage squamous cell carcinoma of the uterine cervix.
Hedman H, **Lindström AK,** Tot T, Stendahl U, Henriksson R, Hellberg D.
submitted

ABBREVIATIONS

CC	cervical cancer
CD4+	immunological marker
CD44	cell-cell adhesion glycoprotein
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CIS	cancer in situ
c-myc	oncoprotein c-myc
COX-2	cyclooxygenase-2
DNA	deoxyribonucleic acid
E	early
EGFR	epidermal growth factor receptor
FCM	flow cytometry
FIGO	Federation Internationale Gynecologie et Obstetrique
HPV	human papillomavirus
HRT	hormone replacement therapy
IHC	immunohistochemistry
Ki-67	proliferation protein
LRIG	leucine-rich repeats and immunoglobulin-like domains
MIB1	monoclonal antibody that detects the Ki-67 antigen
OC	oral contraceptives
OR	odds ratio
PCR	polymerase chain reaction
p16, p21, p27, p53	tumor suppressor proteins
pRb	retinoblastoma protein
RH	radical hysterectomy
RT	radiotherapy
SCC	squamous cell carcinoma
Se-E2	serum estradiol
Se-P	serum progesterone
SPF	S-phase fraction
TMA	tissue microarray
VEGF	vascular endothelial growth factor

INTRODUCTION

Hippocrates of Cos (about 400 B.C.) believed that cancer of the cervix (uterus) was incurable (1). The early idea that cervical cancer was in some way related to matters such as reproduction, marriage, and sexual intercourse was put forward by Rigoni-Stern in 1842 (2). In the 1970s and early 1980s Harald Zur Hausen and his research team made observations that linked human papillomaviruses (HPV) to cervical cancer (CC), for which he was awarded the Nobel prize in medicine in 2008 (3, 4). The introduction of the Papanicolaou (Pap) cervical smear test by George Papapanicolaou in the1920s reduced the incidence and mortality rates of cervical cancer (5, 6). In countries with well functioning screening programs, cytological screening with Pap smear has reduced CC mortality by 40-80 % (7). The incidence of CC has been lowered and more cases have been diagnosed in early stages. However, it will be a long time before there is any likelihood that this cancer will be eradicated. There is still a need for better prognostic prediction to enable a better choice of treatment which reduces adverse effects, saves fertility and ovarian function, and improves survival.

Epidemiology

Cervical cancer is the second most common cancer among women worldwide (Fig.1), with nearly 500,000 new cases per year (8).

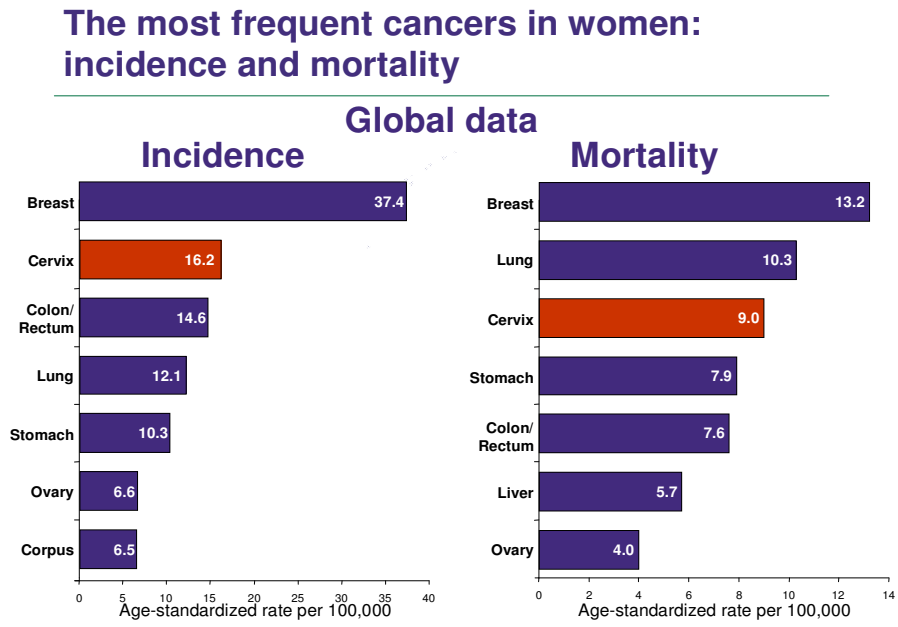

Figure 1. J. Ferlay, F. Bray, P. Pisani and D.M. Parkin. GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide IARC CancerBase No. 5. version 2.0, IARCPress, Lyon, 2004.

Incidence

Substantial regional differences in the incidence and mortality of CC are observed. The incidence varies geographically from 7/100,000 to 43/100,000 (9, 10). The highest incidence is observed in developing countries, while the incidence in industrialized countries has decreased by approximately 60% during the past decades, as a result of screening programs with early detection and treatment of precursor lesions – cervical intraepithelial neoplasia (CIN). Compared to many other cancer types, cervical cancer strikes many women at a relatively early age (11).

Mortality

It is estimated that 274,000 deaths per year can be attributed worldwide to cervical cancer, which globally is the third most common cause of cancer-related death among women. The overall mortality is 55% (8). Mortality rates vary from low-risk regions with good prognosis, to developing countries, where many cases present at a relatively advanced stage (9, 12). Some countries where access to health care is not available report very high mortality rates, such as 80% in Sub-Saharan Africa. Such countries also have among the highest rates of cervical cancer in the world (10, 13).

Etiology

Human papillomavirus

Human papillomavirus (HPV) infections are the most common genital infections worldwide. They are sexually transmitted, in general clinically silent and self-limiting. Some women remain persistent carriers of the viral infection and will be at risk for progression to precancer and cancer of the cervix and, in a lower degree of vulval, vaginal and anal cancer (8, 14). A key factor in progression is the ability of HPV to evade the immune system and establish a persistent infection. HPV infections are associated with penile and anal cancer in men and have also been detected in oral, esophageal, lung, laryngeal, and stomach cancer (8, 15). In both men and women an increased incidence of tonsillar cancer has been reported, and there is an increased proportion of HPV in these tumors (16, 17).

HPV infects epithelial tissue of the skin and the genital mucosa. Eighteen of more than one hundred HPV types are considered to be correlated to cervical cancer and among them 15 are high-risk types and three are moderate-risk. Globally, HPV 16 and 18 are the predominant oncogenic types, which together account for over 70% of all invasive cervical cancer cases (18). High-risk types (hr-HPV) cause precancerous stages and invasive cancer. These have been identified in up to 99.7% of all cases of cervical carcinomas (18-21). Up to 80% of sexually active women will be infected with HPV at some point in their lifetime (22, 23). Most HPV infections are transient. Persistence of HPV infection is a key requirement for progression of the disease. Protein products of the early HPV genes E6 and E7 are responsible for transforming and immortalizing cells,

but they are normally not produced by the virus. Viral proteins E6 and E7 interact with central molecules in cell cycle control. By binding and inducing degradation of p53, HPV E6 proteins inhibit the p53 mediated DNA repair and apoptotic response, resulting in tumor progression. This is known as the most important event in HPV-associated carcinogenesis (24). The unscheduled cycle progression is further enhanced by HPV E7 protein-mediated binding and inactivating pRb. Cells lacking functional p53 and pRb are highly prone to reduce the apoptotic response and increase genomic instability and the proliferation rate, all of which are hallmarks of malignant transformation (25).

HPV is referred to as a necessary but not sufficient factor for invasive cervical cancer (26). Persistent HPV infection seems to be able to lead to preinvasive cancer (CIN III – cancer in situ) on its own, but cofactors are required for development of invasive cancer (20, 27, 28).

Cofactors

Immortalization of the cervical cell is necessary for progression of CIN to invasive cancer. Integration of viral DNA to the host genome that enables expression of viral oncogenes E6 and E7 is a necessary step in immortalization and probably does not occur without the presence of cofactors. *In vitro* and animal studies using cell cultures immortalized by HPV have, with few exceptions, failed to demonstrate progression to invasive cancer, but only to CIN (29). Several possible cofactors have been studied, but interest has been focused on the role of smoking and female sex steroid hormones, e.g. oral contraceptive (OC) use and parity (22, 30-32).

Smoking

Smoking began to be established as a cofactor in cervical cancer with the appearance of epidemiological studies that adjusted for sexual risk behavior (33-35). The first biological evidence of an etiological role of smoking in cervical neoplasms was the finding that levels of nicotine, and its major metabolite cotinine, were increased forty-fold and four-fold, respectively, in the cervical mucus of women with CIN, compared to serum levels (36). Later, benzo (a) pyrene and tobacco-specific nitrosamines were identified in the cervical mucus of smokers, but not of non-smokers (37, 38). In the 1990s smoking was also found to be associated with increased DNA damage in cervical epithelium irrespective of concomitant HPV infection (39, 40).

Tumor marker expression in CC biopsy specimens from smokers has rarely been studied, and then only using a single marker (41). *In vitro* HPV- immortalized cervical cell lines have been treated with smoke condensate and shown to induce cancer (42). The origin of cervical cancer in the transitional area of adenomatous and squamous cell epithelium has been known for many decades. Failure to induce carcinogenesis with smoke condensate in HPV-infected cells from outside the transformation zone has provided evidence of the susceptibility of cells from the transformation zone (43).

Female sex steroid hormones

Oral contraceptives

For more than 20 years, numerous epidemiological studies have almost uniformly found significant associations between cervical neoplasia and long term (in general defined as more than 4-5 years) OC use (44, 45). The epidemiological correlation between long-term OC use and cervical neoplasias was established, and OC use became widely accepted as a risk factor in the mid-1980s, when a number of studies were able to control for sexual risk behavior (46, 47).

Multiparity

High parity has been found in many studies to be associated with cervical cancer and cancer in situ (CIS) (47). Women who have had three or more full-term pregnancies have an increased risk of developing cervical cancer (48). In the IARC-pooled analysis, the odds ratio (OR) for CC in women with seven or more full-term pregnancies was four times higher than that in nulliparous women, and the risk increased linearly with an increasing number of pregnancies (49). Increased levels of estrogen and progesterone induced by pregnancy may modulate the immune response to HPV and influence the risk of persistence or progression (49, 50).

Progesterone

Some laboratory experiments support the epidemiological findings of an association between female steroid sex hormones, in particular progesterone, and HPV-related cervical cell transformation (51-55). It is not known whether increased cell transformation occurs *in vivo* when serum progesterone levels are high, such as during pregnancy and in women with higher natural progesterone levels than the average, or with gestagenic contraceptives (31).

Estradiol

There might be a role for estrogens in CC. When transgenic mice expressing HPV 16 were treated with estrogens, squamous cell carcinomas developed exclusively in the transformation zone (56). Furthermore, it was found in one study that estrogen stimulated differential transcription of human papillomavirus type 16 in SiHa cervical carcinoma cells (57).

Other cofactors and risk factors

Some risk factors are also etiological or cofactors for cervical cancer, such as infection with oncogenic HPV types, smoking, long-term OC use, genetic factors, and impaired host-immune response. Indirect risk factors seem to vary between different geographical regions and between studies.

Early age at first intercourse, many sexual partners, or many sexual partners of the spouse are considered a risk of harboring an HPV infection (47). In some studies previous chlamydial infection has been found to be correlated to increased risk of cervical cancer. It is unclear whether this might be a surrogate for HPV infection or whether chlamydial infection is a cofactor in itself (58, 59).

Women who have never or rarely undergone cervical cytology are at increased risk of developing CC, mainly because screening detects CC precursors and treatment will be given before they have progressed to invasive cancer (60, 61). Among African Americans in the United States the incidence rate for cervical cancer is almost twice the national average. Hispanics and American Indians also have incidence rates above the average (62, 63). Whether this reflects nonattendance at screening, lifestyle differences, or genetic causes is unclear. Women with a low socioeconomic status are at increased risk on account of lack of access to adequate health care services, such as cervical screening (64). Diets low in fruit and vegetables are associated with an increased risk of cervical cancer (65). Several reports have shown that women with impaired immune defence, such as those with human immunodeficiency virus (HIV) infection or being treated with immune-suppressing drugs after a transplant are more likely to develop cervical cancer, and CIN may progress into invasive cancer more rapidly than is usual in the natural history (66). Genetic susceptibility factors also influence the risk of developing cervical cancer. Behavior and personality are influenced by genetic factors (67). Possibly the level of sexual activity is related to genetic factors. A hereditary component has been indicated in comparisons of twins (68). If the mother or sister has had cervical cancer, the risk of developing the disease increases two- or threefold. It is difficult to determine whether this is due to a similar lifestyle or to genetic alterations in common (69, 70). Biological first-degree relatives of women with cervical cancer have an increased risk of developing the disease compared to non-biological (adoptive) first-degree relatives (71). The human leukocyte antigen (HLA) class II has been confirmed as a genetic susceptibility factor for cervical cancer in a population-based cohort of affected sib-pairs (72). There are indications that three chromosomal regions, 9q32, 12q24 and 16q24 contain susceptibility genes with a low to moderate effect on the genetic risk (73).

HPV infection, and in general smoking, long-term OC use and immunodeficiency are the only risk factors that have consistently shown correlation to cervical cancer in independent studies. Genetic factors interacting with HPV are likely to have an impact on the genetic risk of cervical cancer (74).

Carcinogenesis

Carcinogenesis is a complex and multistep process, which involves malignant transformation, insensivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion, and metastasis (25, 75). Each of these processes is the result of numerous cellular events that are not yet fully understood. Tumorigenesis is not the result of a single gene alteration but of a number of events.

HPV infection, in particular infection with HPV 16 and 18, has been associated with more than 90% of cervical cancers. Inactivation of p53, either by interaction with the HPV 16 E6 oncoprotein or by gene mutation in the absence of the virus, appears to be a key step in cervical carcinogenesis. In cervical carcinogenesis three major events have been identified: firstly, the oncogenic effects of E6 and E7 proteins; secondly, the integration of HPV DNA in chromosomal material in the host genome occuring frequently at chromosomes 8 and 12; and thirdly, other genetic alterations not related to HPV seem to be involved (32).

Polymorphism in the human genome can affect the rate of incidence of cancer. Storey *et al* reported that women with the p53codon 72 arg/arg genotype, are at increased risk of developing cervical carcinomas (76).

Two cell types cover the cervix, namely endocervical glandular epithelium and ectocervical squamous epithelium. This ecto-endocervical junction, called the transformation zone, is characterized by transitional pithelium and is the zone where most cervical cancers originate (77).

Invasive squamous cervical cancer (SCC) is preceded by neoplastic precursor lesions, designated cervical intraepithelial neoplasia, low grade (CIN I) and high grade (CIN II-III). CIN III is also referred to as CIS (cancer in situ). CIN I is designated LSIL (low-grade squamous intraepithelial lesions) and CIN II-III is named HSIL (high-grade squamous intraepithelial lesions). Abnormal squamous cells that cannot be classified as LSIL or HSIL are grouped as ASCUS (atypical squamous cells of unknown significance) (78).

Biological markers

Numerous serological and tissue prognostic markers for cervical cancer have been evaluated, often in single studies. Most of these markers have been detected in recent years. Despite promising results for different markers, few have been thoroughly evaluated and confirmed. The prognostic markers are linked to intra- or extracellular mechanisms involved in cancer development. The main mechanisms of those that have been investigated are cell-cell interaction, extracellular space proteins, membranous factors, cell proliferation, neoangiogenesis, tumor hypoxia, immunological factors, HPV interactions, oncogenes, tumor suppression genes and apoptosis (programmed cell death). These markers might be involved in more than one of these mechanisms, but for practical purposes are presented below with respect to what are believed to be their major effects.

Tissue markers

The increasing number of proteins recognised as being up- or down-regulated during carcinogenesis or tumor progression has led to the development of a large number of immunohistoichemical markers representing promising clinical adjuncts. Expression of a specific tumor marker might be clinically useful for prognostic information, cancer screening, staging, early indication of relapse, suspicion of metastases, diagnostic aids, choice of treatment, treatment monitoring, evaluation of novel treatments, dietary supplementation or for other purposes.

A large number of expressed individual biomarkers, often novel or chosen to confirm previous results, have been proposed as being of possible prognostic value in cervical cancer, often enthusiastically in initial reports, but subsequent studies have frequently shown disappointing or contradictory results (79). No systematic approach in selecting biomarkers that influence different steps of cervical carcinogenesis has previously been attempted in cervical cancer. In the present research expression of 11 tumor markers has been investigated regarding their ability for prognostic prediction and their correlation to cofactors.

Tissue markers in the present study

Proliferation: EGFR and Ki-67

EGFR

Epidermal growth factor receptor (EGFR) and Ki-67 are two of the most widely investigated proliferation factors in cancer and have been associated with a poor prognosis in a number of cancer types (80). EGFR is also one of the most investigated prognostic markers in cervical cancer. EGFR interacts with specific cell-surface receptors and transduces intracellular signals to stimulate DNA synthesis and cell division. Evaluations of EGFR expression as a prognostic marker for cervical cancer have yielded conflicting results. In some studies no relationship to overall survival was found (81, 82). Other studies, however, have shown EGFR to be correlated with a poor prognosis, even in multivariate analyses (83-86).

Ki-67

The monoclonal antibody Ki-67 (MIB-1) detects a nuclear antigen that is present only in proliferating cells. The Ki-67 antigen is present in proliferating cells but absent in resting cells (the Ki-67 antigen is expressed in all stages of the cell cycle except G0 and early G1 phases) (87). Ki-67 has been found to be more intensely stained in HPV-positive than in HPV-negative epithelium (88, 89). Expression of Ki-67 has been thoroughly investigated in invasive cervical cancer but the association between this marker and the prognosis is still controversial (85, 90-94).

Oncoproteins: c-myc

c-myc

c-myc is one of the 'classic' oncogenes, and its translocation in Burkitt´s lymphoma was first demonstrated in 1982 (95, 96). The functions of c-myc products are still not completely understood, as c-myc binds to hundreds of potential target genes. It is evident, however, that c-myc expression contributes to increased proliferation and loss of differentiation (97). c-myc expression has been found in many studies to be associated with poor survival in cervical cancer, although there are discrepancies in the results (98, 99).

Tumor suppressor proteins: p53, p27 and LRIG1

p53

Tumor Suppressor Protein p53 is a nuclear phosphoprotein encoded by the p53 gene, whose normal function is to control cell proliferation and apoptosis. p53 regulates the response of cells to DNA damage by causing apoptosis or cell cycle arrest at the G1 and G2 checkpoints prior to DNA replication allowing repair of damaged DNA. It thereby functions as a tumor suppressor and hampers development of cancer cells (100). Mutations of the p53 gene are frequently found in most invasive cancers, resulting in loss of the tumor suppressor functions of wild type (normal) p53 and gain of oncogenic functions. Mutant p53 is frequently found in invasive cancers. Appearance of mutations of the p53 gene seems to be a late event in cervical cancer and has not been shown to be a prognostic marker. In cervical cancer mutated p53 is rare, as it is degraded by HPV E6 oncogene (101). Overexpression of p53 has been thoroughly studied as a possible prognostic marker for cervical cancer (102-104).

p27
p27 is a p53 related protein with a tumor suppressor capacity that blocks cell cycle proliferation in the G1-S phase by inactivation of cyclin/cdk. The p27 protein is also involved in the regulation of cell migration and mitosis (105-107). Results regarding expression of p27 in SCC have been conflicting (108, 109) .

LRIG1
The leucine-rich repeats and immunoglobulin-like domains (LRIG) family includes three glycoproteins that were identified at the Oncology Laboratory in Umeå. In this study we focused on LRIG1 (Fig. 2).

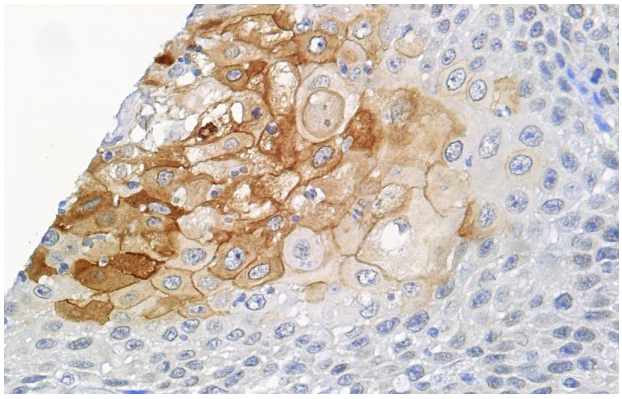

Figure 2. Immunohistochemical staining of LRIG1. Courtesy of Hedman H, Henriksson R, Department of Radiation Sciences, Norrland University Hospital, Umeå, Sweden.

A large number of organs were studied by immunohistochemistry and LRIG1 was expressed in normal tissue of most body organs, but there was no expression in the uterine cervix. LRIG1 mRNA, however, seems to be expressed there. LRIG1 suppressed EGFR (also called ErbB1 or Her1) by downregulation and was thus a potential tumor suppressor (Fig. 3). This was supported by the finding that perinuclear expression of LRIG proteins correlated with survival in patients with astrocytic tumors (110, 111). LRIG1 expression and its possible clinical implication have not been studied previously in cervical cancer.

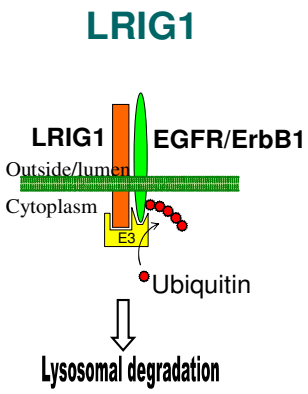

Figure 3. LRIG1 acts as a negative regulator of ErbB1/EGF receptor signaling by enhancing receptor ubiquitylation and lysosomal degradation. Adapted from Hedman and Henriksson, 2007, Eur J Cancer 43:676.

Neoangiogenesis: VEGF

VEGF

The growth of tumors beyond 1-2 mm is dependent on the formation of new blood vessels, neoangiogenesis. Vascular endothelial growth factor (VEGF) is a cytokine that serves a central function as a key regulator of physiological and pathological angiogenesis (112). It has been correlated to poor prognosis in some studies of cervical cancer (84, 113). In one study the intensity of VEGF expression, but not the area that was positively stained, correlated to poor prognosis in multifactorial analyses (114).

Cell-cell adhesion: Cadherins and CD44

E-cadherin

The processes of invasion and metastasis are complex. The escape of cancer cells from the primary tumor involves disruption of normal cell-cell adhesion. Epithelium cadherin (E-cadherin) is one of the most studied cell adhesion molecules. In normal tissue cell-cell interaction is dependent on various extra cellular matrix proteins that function as cell adhesion molecules. With progression of cervical neoplasia, E-cadherin seems to lose its expression on the cell membrane and gain expression in the cytoplasm, i.e. a diffuse expression appears (115, 116). Increased cytoplasmic staining has also been observed with worsening differentiation in invasive cervical carcinomas (117). Loss of E-cadherin expression was significantly associated with reduced overall survival and disease-free survival in early stage SCC in one study (118).

CD44

CD44 is a widely studied cell adhesion molecule. Aberrant expressions of CD44 isoforms (splice variants) have been reported to be associated with poor prognosis in a variety of cancers (119). In invasive cervical cancer CD44 has been reported to be correlated with a poor prognosis (120, 121). However, in an experimental study where different cervical cancer cell lines were compared with normal cervical tissue, no difference in expression was observed (122).

Immune response: CD4+

CD4+

The immune response to cancer is mainly dependent on cell-mediated immunity such as the presence of CD4+ and CD8+ T-cells and natural killer (NK) cells. The presence of a large number of NK cells, and increased serum CD4+ and CD8+ T-cell levels, have been associated with a favorable response in patients with cervical cancer treated with neoadjuvant chemotherapy (123).

Multiple mechanisms: COX-2

COX-2

Apoptosis activity balances cell proliferation. Decreased apoptosis or increased proliferation leads to tumor growth, and apoptosis inhibitors thus contribute to the growth of tumors. One such inhibitor is cyclooxygenase-2 (COX-2), which is related to prostaglandin secretion (Fig. 4). In addition, COX-2 is associated with the inflammatory response to tumors, tumor invasion, and neoangiogenesis (124). Recent studies have demonstrated that COX-2 expression is up-regulated in a number of cancer types (125-127).

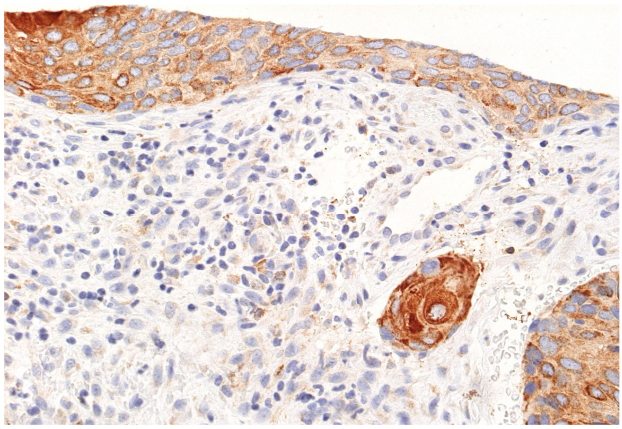

Figure 4. Immunohistochemical staining of COX-2, cytoplasmic expression. Courtesy of Tibor Tot, Department of Pathology, Central Hospital, Falun, Sweden.

Other major tissue markers

Retinoblastoma susceptibility gene

Rb1 or Rb was one of the first tumor markers to be detected and located on chromosome 13q14.2. The Rb gene is mutated in hereditary retinoblastoma. It encodes a nucleoprotein (pRb) that plays a key role in the regulation of the cell cycle, where it controls the G1–S transition (128, 129). pRb inhibits the E2F family of transcription factors, which activate transcription of genes required for DNA replication (25).

$p16^{INK4A}$ and $p21^{WAF1/CIP1}$

Expression of the cycline-dependent kinase (cdk) inhibitors p16 and p21 has recently aroused interest. Cdk are protein complexes that are related to progression through the cell cycle. Absence of p16 protein expression in normal squamous cervical epithelium and increased expression in SCC, support the hypothesis that the HPV 16 E7 oncoprotein inactivates Rb (130). The effects of p21 knockout in mice and its expression patterns in human cancer are consistent with a role for p21 as both a tumor suppressor and an oncogene (131). In one study none of the proteins p16, p21, or p27 were independently associated with prognosis in stage IB SCC (132).

Serum markers

Serum tumor markers seem to have a limited role as prognostic factors in cervical cancer, but are sometimes used at follow-up, after treatment. Squamous cell carcinoma antigen (SCC-Ag), tissue polypeptide antigen (TPA) and Cyfra 21-1 are serum markers used in squamous cell cervical cancer. In one study SCC-Ag was found to be useful in predicting complete remission after treatment, while serum TPA and Cyfra 21-1 were less useful (133). In another study by the same group the three tumor markers were of no value in predicting lymph node metastases and prognosis (134). Other serum markers used for follow-up in a number of cancers, such as CEA and CA 125, have a minor role in predicting prognosis (135, 136). Serological tumor markers may be useful for an earlier discovery of recurrence of adenocarcinoma but have a limited role as prognostic factors in squamous cell cervical cancer (137, 138).

DNA cytometry − S-phase fraction (SPF) and ploidy

The nuclear DNA content (DNA ploidy) and the proportion of cells in division (S-phase fraction) have been shown to be of value as prognostic markers in carcinomas (139, 140). Flow cytometry (FCM) is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them through an electronic detection apparatus. An increased cell proliferation rate is necessary for tumor growth. The proportion of cells in a tissue sample with a DNA content between that of cells in G0/G1 and G2 of the cell cycle constitutes the S-phase fraction (SPF). The S-phase of the cell cycle is a well-known and studied measure of proliferation. The S-phase fraction and ploidy level are determined by flow cytometry (141). Cervical cancer studies on correlation between flow cytometric parameters and prognosis have shown conflicting results (142-144).

Histology

Histological type

Carcinomas of the cervix are categorized on morphological grounds into squamous cell carcinomas (75.9 %), adenocarcinomas (11.4 %), adenosquamous carcinomas, neuro-endocrine tumors, and others (12.7 %) (145, 146).

Histological grade

Squamous cell cervical carcinoma tumors are also graded based on their degree of differentiation into well-, moderately-, poorly-, or undifferentiated. Histological grade, as currently assessed, is of little clinical value (147).

Stage

Cervical cancer is clinically staged in accordance with the system of the International Federation of Gynecology and Obstetrics (FIGO) (Fig. 5). Classification is performed prior to treatment and based on clinical examination including examination under anesthesia, colposcopy, biopsy, endocervical curettage, hysteroscopy, cystoscopy, proctoscopy, intravenous urography and X-ray examination of the lungs and skeleton (148). In 2009, FIGO modified the staging system by dividing Stage IIA into substages. Stage IIA1: tumor size of less than or equal to 4 cm with involvement of less than the upper two-thirds of the vagina. Stage IIA2: tumor size of more than 4 cm with involvement of less than the upper two-thirds of the vagina. For institutions with access to MRI/CT scanning, radiological tumor volume and parametrial invasion should be recorded. Examination under anesthesia, cystoscopy, proctoscopy, and intravenous urography are no longer mandatory. The redefined stages have been effective from January 2009 (149).

Stages of cervical cancer

Stage	5-year survival, %	Description
Ia	90–100	Cancer has invaded cervix but not spread
Ib	80–90	Cancer may be seen without a microscope – may have spread > 5 mm deep or > 7 mm wide
IIa	75	Cancer spread beyond cervix to upper vagina, not lower third of vagina
IIb	50–60	Cancer spread to parametrial tissue next to cervix
IIIa	20–40	Cancer spread to lower third of vagina, but not to pelvic wall
IIIb	20–40	Cancer extends to pelvic wall and/or blocks urine flow to bladder
IVa	5–10	Cancer spread to bladder or rectum
IVb	0	Cancer spread to distant organs, e.g. lungs

FIGO Committee on Gynecologic Oncology. Benedet JL, *et al. Int J Gynaecol Obstet* 2000; **70:**209-262.

Figure 5. Clinical stages of cervical cancer.

Prevention

Primary prevention

Reducing infection is one way of primary prevention. The use of condoms and the ABC concept used for HIV prevention in Uganda (Abstinence, Be faithful, use Condoms) are ways of reducing the incidence of high risk HPV infection (150).

Prophylactic Vaccines

Prophylactic HPV vaccines, which generate neutralizing antibodies in order to prevent infection, have been developed. Two vaccines have recently been licensed in many countries. Both use virus-like particles (VLPs) consisting of recombinant L1 capsid proteins of individual HPV types to prevent HPV 16 and HPV 18 induced precancerous lesions and cancer (151, 152). Vaccines against HPV 16 and 18 probably decrease the future risk of invasive cervical cancer if given during adolescence and preferably before the first intercourse (153). As the latency period from HPV infection to invasive cancer is a matter of decades, a rapid decrease in incidence cannot be expected. Cross protection against related oncogenic HPV types will probably improve the efficiency further, but vaccination will not completely eradicate cervical cancer, bearing in mind that 18 high-risk HPV types are known at present. The importance of continued use of cytological screening and/or HPV tests in both vaccinated and unvaccinated women cannot be emphasized enough.

Secondary prevention: Cytology, colposcopy, VIA, VILI and HPV test

Cytology (Pap smear) is used in screening programs for the identification of precursor lesions (CIN) in cervical smears. Screening will be the main preventive method for the current generation of women aged 20 years and older and screening will be necessary also for younger generations, as prophylactic vaccines have limitations. The screening program has been successful and in Sweden it has decreased the incidence of cervical cancer by about 50% (154). The HPV test as a screening tool for CIN has been under evaluation during recent years (155, 156). Pap smear testing alone is an insufficient diagnostic tool, since its sensitivity is at best between 50 and 75% (up to 94 % if Autopap-directed rescreening or thin layer methods are used) (157). Cytology is less sensitive in older women. It has been proposed that a test for high-risk HPV may have a higher sensitivity and specificity in middle-aged and older women and might be an alternative to cytology as a tool in screening (158, 159). In some areas without resources for screening with cytology (Pap smear), HPV testing or colposcopic diagnosis with inspection of the cervix is performed. Visual inspection with acetic acid (VIA) and visual inspection with Lugol´s iodine (VILI) have been implemented but show several weaknesses, particularly a high rate of false positive findings (160).

Treatment

Traditionally in many regions of Sweden, the treatment of cervical cancer has been radiotherapy. During 1984-90, the period when patients were included in this study, surgery in early stages of cancer combined with radiotherapy was introduced into cancer treatment in the northern region of Sweden.

Current and future treatment of cervical cancer includes surgery, radiotherapy, chemotherapy, and pharmaceuticals directed towards tumor markers (161). In early stages the treatment of cervical cancer is surgery, sometimes in combination with radiotherapy and chemotherapy. Later stages are treated with radiotherapy and chemotherapy. The classical surgical management of early-stage cervical carcinoma, known as radical hysterectomy (RH), was first described by Wertheim more than one hundred years ago and was then modified by Meigs in the 1950s. Radical hysterectomy with minor modifications is still the mode of surgical treatment today. During the last two decades, new surgical operations -radical trachelectomy, nerve-sparing radical hysterectomy, laparoscopic assisted radical vaginal hysterectomy, robot assisted laparoscopic radical hysterectomy, laparoscopic lumbo-aortic lymph node dissection, and laparoscopic pelvic exenteration- have been proposed for the management of cervical carcinoma.

Microinvasive cancer (stage IA) is usually treated by hysterectomy including the upper part of the vagina. For stage IA2, the lymph nodes are also removed. For patients who wish to remain fertile, an alternative can be a local surgical procedure in which a cone-shaped piece of the cervix is removed (conization). Another possible treatment option for patients who want to preserve their fertility is a trachelectomy. In this, an attempt is made to remove the cervix surgically while preserving the ovaries and uterus. It is also an option

for those in stage IB cervical cancer with small tumors. A radical trachelectomy can be performed abdominally or vaginally (162). Women who are able to conceive after surgery are susceptible to preterm labor and possible late miscarriage. Early tumors, stages IB1 and IIA1, can be treated with surgery (RH with removal of the lymph nodes) or chemoradiation therapy. Radiation therapy is given as external beam radiotherapy to the pelvis and brachytherapy (internal radiation). Patients treated with surgery in whom high-risk features are found on pathological examination are given radiation therapy with chemotherapy in order to reduce the risk of relapse. Tumors of stages IB2 and IIA2 may be treated with radiation therapy and cisplatin-based chemotherapy, hysterectomy (which then requires adjuvant radiation therapy), or cisplatin chemotherapy followed by hysterectomy. Advanced stage tumors (IIB-IVA) are treated with radiation therapy and cisplatin-based chemotherapy.

In early cervical cancer identification of tumor spread to regional lymph nodes is essential for adequate treatment and to provide prognostic information. Studies have indicated that the pelvic sentinel node status may accurately predict the state of the regional lymph nodes (163-165).

Therapeutic vaccines

A number of therapeutic vaccines targeting E6 and E7 have been developed. These vaccines aim to control HPV infection through cell-mediated immunity and have shown promising results in both preclinical and clinical trials (166).

Prognosis

The prognosis in women with cervical cancer varies. In countries with screening programs and well functioning health care for the whole population, where a high proportion of women are diagnosed in early stages and treated with surgery/radiation and cytotoxics, the survival rate is 60-70 % (Fig. 6), compared with 21% in Sub-Saharan Africa (9, 13, 167, 168).

Clinical prognostic factors

At present, lymph node status and clinical stage are the most important prognostic factors in cervical cancer. Clinical staging is traditionally the most commonly used predictive factor and is also the most important single parameter influencing choice of treatment. The variation in tumor volume within the same stage might be equally important in early stages and in locally advanced stages. Microinvasive carcinomas with stromal invasion less than 3 mm in depth have only a minimal risk of having lymph node metastases. Depth of invasion, distant metastases, and vascular space invasion are other important independent histopathological indicators of prognosis that have been used to choose tailored treatment for the individual patient (169-172).

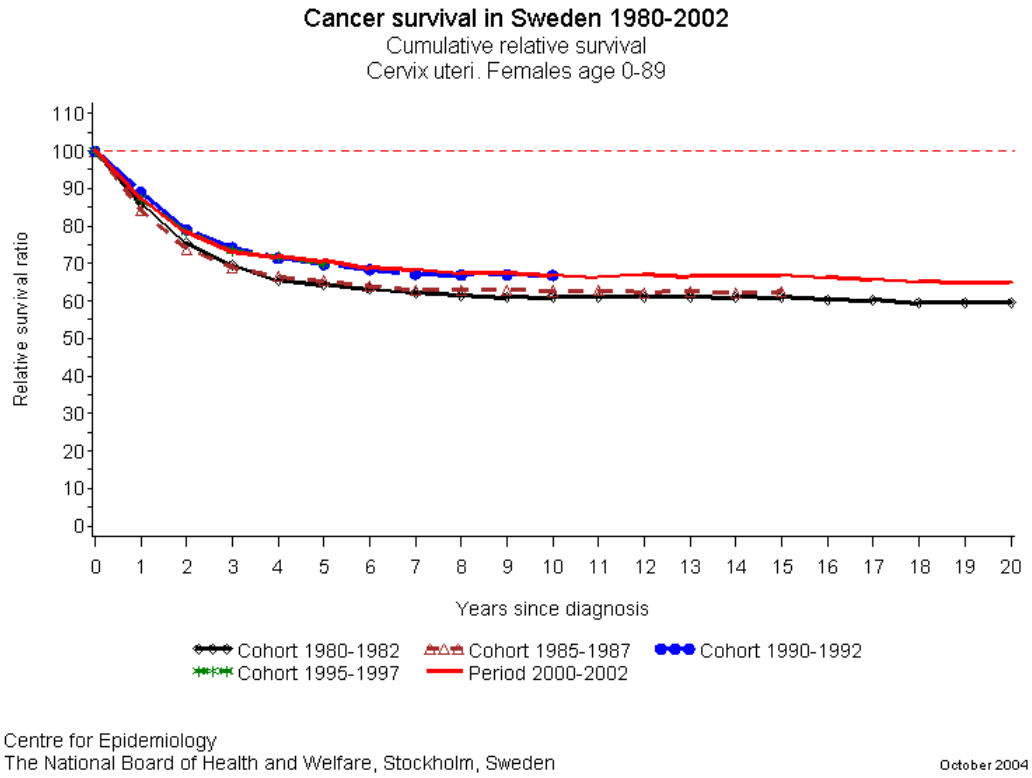

Figure 6. Cumulative relative survival, cervix uteri, Sweden 1980-2002**.**

Other prognostic factors

Apoptotic index

Increased apoptosis has been found to be correlated to the level of tumor proliferation and aggressiveness, and the apoptotic index also to tumor size and hypoxia, and these have been used together with clinical factors to predict the prognosis. A high apoptotic index has been associated with poor local control and long term prognosis in advanced squamous cell carcinoma(173).

Malignancy grading systems

Multifactorial malignancy grading systems have also been proposed, but their clinical usefulness has been limited (174, 175). Prognostic indicators, in addition to clinical findings, are thus warranted, such as expression of cancer-associated proteins and genes (79).

HPV

HPV is found in practically all cervical cancers and is thus not useful for prognosis. HPV type has not been associated either with survival or with morphological types of cancer (176). Neither has expression of HPV 16 antibodies to E2, L2 and E7 peptides (ELISA) been shown to be of prognostic importance for recurrence, nor has the presence of antibodies to E6 and E7, the major oncoproteins, correlated to prognosis (177, 178). Although these and other oncoproteins are important in the pathways of cell transformation and cancer development, they do not seem to be important for prognostic prediction.

AIMS OF THE INVESTIGATION

Overall aim:
to study possible correlation between female sexual steroids, smoking, SPF, and tumor marker expression, and their role in prognostic prediction in squamous cell cervical cancer.

Specific aims:

- to investigate possible correlation of tumor growth, measured as S-phase fraction, with the concentrations of serum progesterone and serum estradiol, smoking habits, as well as prognosis in squamous cell cervical cancer;

- to assess the length of survival in women who died from cervical cancer in relation to their serum progesterone and estradiol levels;

- to address the question whether combinations of ten different prognostic markers, relevant in cervical cancer and representing different steps in carcinogenesis, might improve the prognostic prediction in invasive cervical cancer;

- to compare the expression of these tumor markers with the smoking status and serum estradiol and progesterone levels in women with squamous cell cervical cancer; and

- to evaluate LRIG1 as a prognosis predictor and its correlations to cofactors in squamous cell cervical cancer.

MATERIALS AND METHODS

Patients

The investigation comprised 190 women with squamous cell cancer (76.9 % of all 247 patients with cervical cancer of stages IB-IV) admitted to the Department of Gynecologic Oncology, Norrland University Hospital, Umeå, Sweden during the period September 1984 to October 1990. The study was prospective. However, during a period in the middle of the study, there was a gap in sampling for serum estradiol and progesterone as well as biopsies for flow cytometry, due to the absence of the relevant investigator. The choice of treatment was the same during the study period, with radiation and/or surgery according to the contemporary routines.

The women were followed up for at least ten years. In cases of any uncertainties regarding causes of death, women were checked against the mortality registry of the National Board of Health and Welfare to avoid any mistakes. All-cause mortality and disease-specific mortality were recorded. The mortality rate in this study is the disease-specific mortality rate.

Clinical staging was performed according to FIGO, and the WHO criteria were applied for histological grading (148, 179).

Analyses for specific studies include flow cytometry on fresh frozen tumor biopsy samples for analyzing S-phase fraction and ploidy, serum samples for analyses of sex steroid hormones, and tumor marker expression using paraffin embedded tumor material (Fig. 7). Pre-treatment blood samples, collected in connection with tumor biopsy for flow cytometry, were taken on admission to the clinic. Tumor biopsy samples for paraffin embedding were obtained before treatment. Occasional data are missing for individual women, but this was not systematic.

Menopause was defined as not having had menstrual-like vaginal bleeding during the previous six months.

For reasons given above, in 128 women both serum progesterone and estradiol were analyzed. In total 148 women had flow cytometry with ploidy analyses and 139 had S-phase fraction estimations.

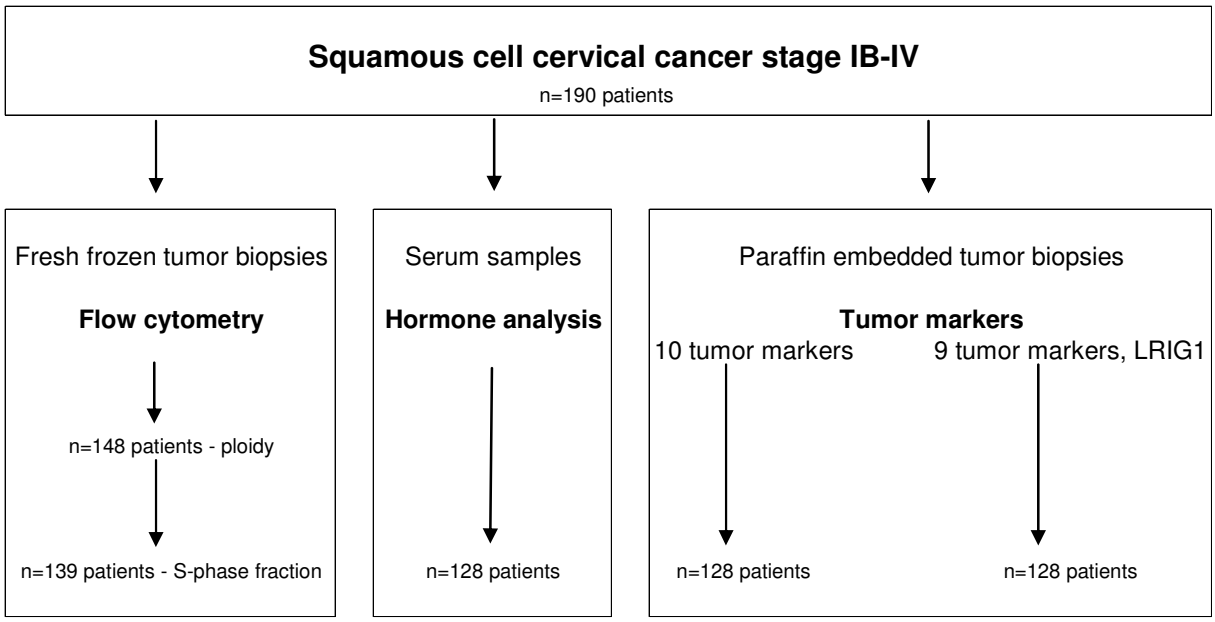

Figure 7. Flowchart for the whole study population.

Tumor marker expression was estimated in 128 patients; these were not, as a group, the same patients as those that underwent hormone analyses. The patients in the group in which ten tumor markers were analyzed were not exactly the same as in the group where LRIG1 and nine tumor markers were analyzed.

General characteristics of the whole study of 190 patients are presented in Table I, and the ten-year mortality rates by clinical stage are given in Table II.

Table I. General characteristics of the study population, 190 patients.

	Study population n=190	
Mean age (SD), years	56.8	17.1
Pregnancies (no.,SD)	2.9	1.9
Parity (SD)	2.5	1.7
Postmenopausal (no., %)	118	62.1
Menarche (mean age, SD), years	13.5	1.6
Menopause (mean age, SD), years	48.1	4.8
Body mass index (mean, SD)	25.0	4.6
Smokers (no., %)	79	41.6
Ever OC (no., %)	69	38.3

Table II. Ten- year mortality rates in the total study population, 190 patients, by clinical stage.

Clinical stage	Patients no.	%	Mortality rate %
IB	85	44.7	21.1
IIA	23	12.1	39.1
IIB	30	15.8	46.7
IIIA	5	2.6	80.0
IIIB	38	20.0	68.4
IV	9	4.7	88.9

Flow cytometry

Fresh frozen tumor biopsy samples for flow cytometry were taken on admission to the clinic. The tumor samples were immediately frozen and stored at -70° until analyzed. A suspension of cell nuclei was obtained using a combined mechanical and enzymatic technique, as described in detail by Tribukait (141).

Ploidy

The DNA content was expressed in relative DNA values, where the DNA content of normal diploid GO/GI cells was given the value 2c. Human lymphocytes with a diploid DNA content were used as an external standard. Tumors with a DNA ranging from 1.8 c to 2.2 c were regarded as peridiploid. Tumors diverging from these values were regarded as aneuploid.

S-phase fraction

The DNA S-phase fraction (SPF) was evaluated by flow cytometry, as a measure of proliferation. SPF was calculated as the proportion of cells between G1 and G2 peaks, corrected for the background fluorescence, according to the simplified method described by Baisch *et al* (180).

Hormone analysis

The serum samples taken on admission to the clinic were frozen and stored at -70° until analyzed. Serum (se) progesterone (P) and estradiol (E2) were measured in duplicate by radio-immunoassay (RIA) after celite chromatography, as previously described (181). The serum estradiol concentration was measured in pmol/L and serum progesterone in nmol/L. The day of the menstrual cycle was recorded at the time of sampling.

Tumor markers

Tumor markers, their functions and localization are described in Table III.

Paraffin embedded tumor biopsy samples

Tumor biopsy samples were taken at diagnosis at the local hospital and on admission to the Department of Gynecologic Oncology, Norrland University Hospital, Umeå, prior to treatment.

Tissue microarray

With the tissue microarray (TMA) technique, tissue cores as small as 0.6-2 mm in diameter, are removed from regions of interest in paraffin-embedded tissues such as clinical biopsy samples or tumor samples. These tissue cores are then inserted in a recipient paraffin block in a precisely spaced, array pattern. Material from up to 1000 patients can be set in one block (182). Sections from this block are cut into thin μm sections set on one glass/slide and then analyzed by any method of standard histological analysis, often immunohistochemistry or in situ hybridization.

Sections of three μm of the original paraffin blocks were reviewed by a senior pathologist (Tibor Tot) and the most representative area was marked for TMA. The microscopic evaluation included the complete TMA-biopsy specimen. Three-millimeter punch biopsies were taken from the blocks corresponding to the marked area and joined into TMA paraffin blocks, each containing an average of 25 punch biopsies. Each TMA block also included two controls, muscle, skin, prostate, ovary or thyroid.

Immunohistochemical analysis

The eleven tumor markers that were evaluated in this study were identified by immunohistochemistry (IHC). IHC analysis refers to the process of microscopic localization of specific proteins in cells of a tissue section by staining with antibodies (183). Immunohistochemical staining is widely used in the diagnosis and treatment of cancer. Specific molecular markers are characteristic of particular cancer types. IHC is also widely used in basic research for determining the distribution and localization of biomarkers in different parts of a tissue (184, 185). IHC staining was carried out at the Department of Pathology and Clinical Cytology, Falun with the Dako Autostainer, which uses biotinylated secondary goat anti-mouse antibody for the detection system and streptavidin-horseradish peroxidase conjugate for visualization of diaminobenzidine (DAB) solution. The slides were weakly counterstained with hematoxylin and were mounted with pertex. Staining for LRIG1 was carried out at the Oncology Laboratory, Umeå.

Eleven tumor markers were chosen. They were selected to represent at least eight different major functions in cancer, i.e., malignant transformation, proliferation, cell cycle arrest (tumor suppression), cell-cell adhesion, apoptosis, angiogenesis, prostaglandin synthesis, and immune response.

Table III. Tumor markers, their functions and localization.

Biological marker	**Functions**	**Localization**
Epithelial Growth Factor Receptor (EGFR)	Proliferation	Membrane
Ki-67 (MIB-1)	Proliferation	Nucleus
C-myc	Cell cycle progression, malignant transformation	Nucleus
p-53	Cell cycle arrest, apoptosis DNA repair	Nucleus
p-27	Cell cycle arrest	Nucleus
E-cadherin	Cell-cell adhesion	Membrane
CD44	Cell-cell adhesion	Membrane
Vascular Endothelial Growth Factor (VEGF)	Angiogenesis	Membrane
Cyclooxygenase-2	Inflammation, angiogenesis, apoptosis	Cytoplasm
CD4+	Immune response	Intercellular
Leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1)	Not established	Nucleus Cytoplasm

Expression of all 11 antibodies was evaluated by an indepent senior pathologist (Anders Lindgren), who was blinded to clinical details. A four-grade semiquantitative score was used, where 0 was absence of biomarker expression, 1 was expression in 1-19% of cancer cells, 2 was expression in 20-49% and 3 was expression in 50% or more cells. For E-cadherin, however, intensity of staining was used for grading (absent, mild, moderate, or intense staining). This was also true for COX-2. Evaluation of intensity was based on the pathologist's long experience of immunohistochemistry. CD4+ was assessed in the area surrounding the cancer cells. For technical reasons there were occasional cases (one to four per biomarker) where an individual biomarker could not be evaluated in individual patients. Staining was assessed in the nucleus, cytoplasm, or cell membrane, as appropriate. Aberrant staining was recorded, but there was no evidence that this was of prognostic or other importance.

As there is no general consensus for cut-off, the best explanatory cut-off level was used when the results of biomarker staining were dichotomized. When there was no evidence of any association between any variables, dichotomization was performed such that similar numbers of patients were included in the two groups. With the exception of VEGF, there were no tendencies for different clinical stages to be associated with different antibody staining. VEGF was expressed in 78% of stages IB/IIA and in 60% of IIB/IV ($p=0.03$).

Statistical analysis

The Chi2 test/Fisher´s exact test for categorical variables, t-test/Mann-Whitney U-test for continuous variables, and logistic regression to estimate odds ratios (OR) and 95% confidence intervals (CI) or to check for and identify possible confounders in multivariate analyses were used when appropriate. Specific analyses will be given for each study below and in the relevant paper. The statistical software used was JMP version 3.1.2, SAS Institute, Cary, NC, USA.

Study I

The study group and exclusions are presented in the flowchart (Fig.8). Biopsy samples of invasive squamous cancer of the cervix uteri of 148 women were investigated by flow cytometry. In 112 cases (77, 2 %) SPF, serum progesterone and serum estradiol were all studied. After exclusion of six women who had had cervical cancer during pregnancy or within six months post partum, and exclusion of three women because of recurrent disease, 103 women were eligible for complete evaluation, see methodological considerations below.

We investigated correlations between serum progesterone and serum estradiol levels, smoking, and DNA S-phase fraction and ploidy respectively.

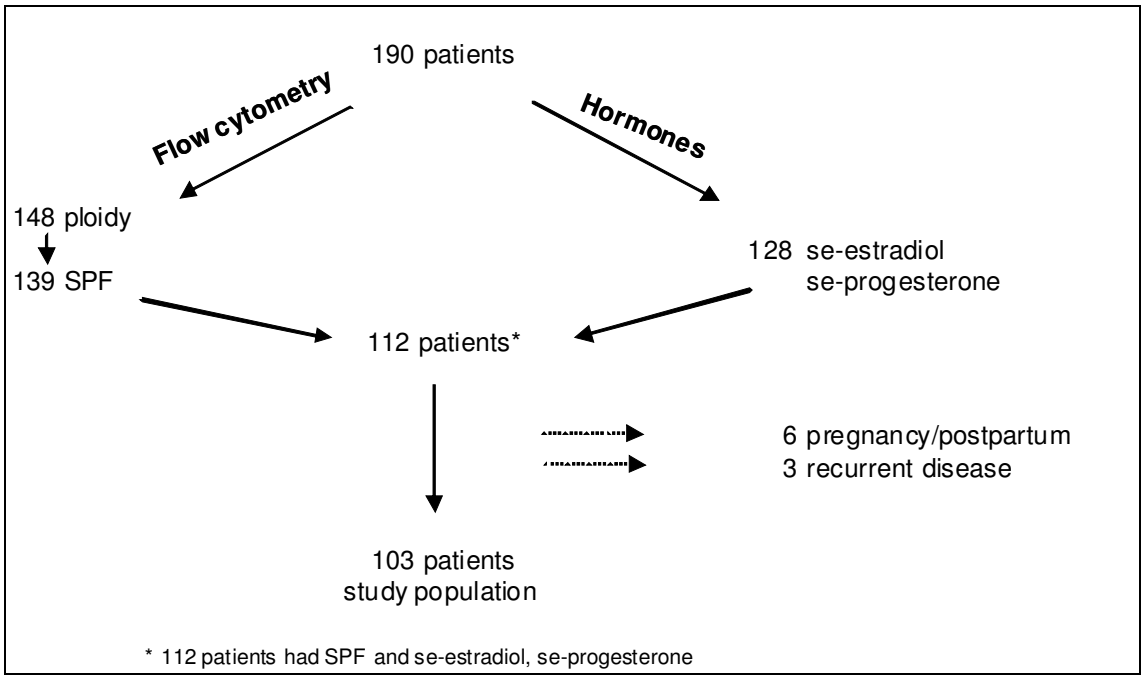

Figure 8. Flowchart for the patients in study I.

Statistics

Crude significance analyses of dichotomous and non-dichotomous categorical variables, such as ploidy level and smoking, were made by Fisher´s exact test. The Mann-Whitney U-test was used for non-dichotomous categorical variables, such as histological grading

and clinical staging. The t-test was used for continuous variables, e.g., age and age at menopause. Hormone concentrations in relation to SPF were studied, using analyses of correlation and linear stepwise regression. Stratification for ploidy level and smoking was used to study the correlations between hormone concentrations and SPF (≥14%) in different subgroups. Logistic regression was applied in multifactorial analyses to check for and identify possible confounders.

Study II

The numbers of women investigated in study II are given in the flowchart below (Fig. 9). Twelve premenopausal women (24%) and 45 of the postmenopausal women (57, 7%) eventually died of their disease and were studied regarding relation of different parameters to length of survival after admission. The evaluation included clinical stage, tumor differentiation, climacteric status, smoking, body mass index (BMI), ploidy, SPF and serum progesterone and estradiol. Variables and laboratory analyses were the same as in study I.

We investigated endogenous estradiol and progesterone levels and SPF in relation to length of survival in women who died of squamous cell cervical cancer.

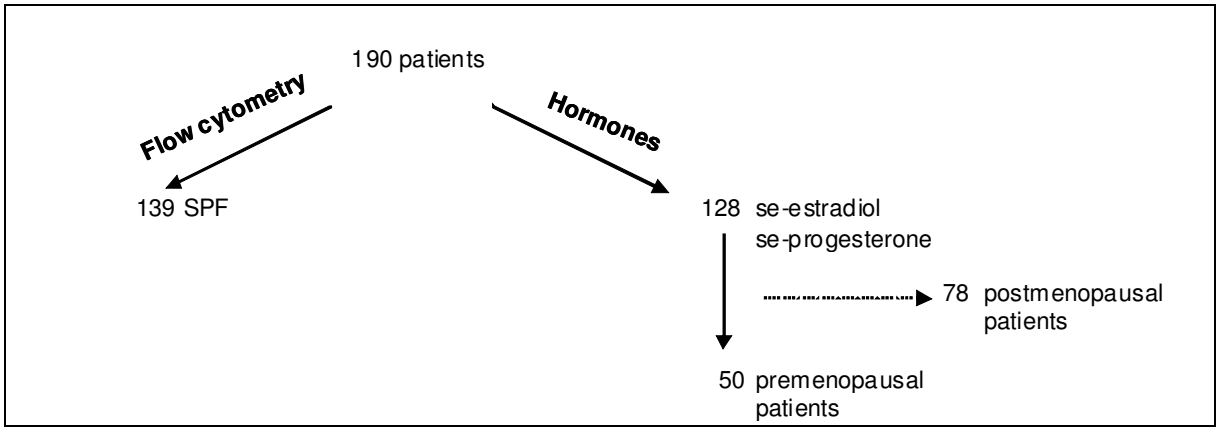

Figure 9. Flowchart for patients in study II.

Statistics

A stepwise increase in cut-off levels for serum hormones, estradiol/progesterone ratio and SPF was used to find the best explanatory cut-off values. The cut-off values chosen were 12% for SPF in the whole study population, and an estradiol/progesterone ratio of 60 (which was also the median ratio) in premenopausal women. Curve fitting and significance testing for two continuous variables, i.e., serum hormone levels and length of survival were analyzed by linear regression. Logistic regression was used in multifactorial analyses to check for and identify possible confounders, i.e., clinical stage and estradiol/progesterone ratio in relation to survival.

Studies III −IV and V

The study population of studies III-IV consisted of 128 women for whom paraffin blocks were available out of the 190 women included in the study. For study V the study population also consisted of 128 women, but not entirely the same women as in study III-IV (Fig. 10). An extensive search was made for the remaining biopsy samples, initially at the four pathological departments in Umeå, Sunderbyn, Sundsvall and Östersund, but they were not stored at those departments. No more paraffin blocks were found through contacts with leaders of research projects in which these had been included.

We evaluated ten different tumor markers, singly and in combination, and the tumor marker LRIG1, as prognostic predictors, in relation to the overall ten-year mortality rate. Expression of these tumor markers was also investigated in relation to smoking habits and to serum estradiol and progesterone levels.

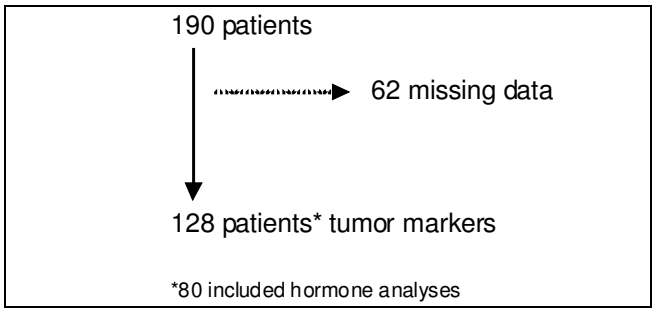

Figure 10. Flowchart for patients in studies III-IV and V. A small number of patients in study V were not included in studies III and IV and *vice versa*. All three studies, however, encompassed 128 patients.

Statistics

Statistical analyses of survival included Kaplan-Meier estimates for survival curves (log-rank test). Cox regression (proportional hazard) was used to analyze temporal trends in survival and the results are given as risk ratio, 95% CI and p-value after adjustment for possible confounders. Overall ten-year survival was analyzed by logistic regression, enabling adjustments to be made for confounding factors to establish OR, 95% CI and p-value. Clinical stage was dichotomized into stage IB/IIA and IIB-IV. For LRIG1 the kappa index was used to calculate the degree of agreement with the other tumor markers. An index of more than 0.40 was considered to represent fair or good agreement.

Methodological considerations

- Studies that evaluate prognostic markers for squamous cell cervical cancer are in general smaller than the present study. A study population with 128 cases of invasive squamous cell carcinoma was shown to be reasonable, as differences between groups should be substantial to be clinically useful.

- In study I, pregnant and post partum women (n=6) and those with recurrent disease (n=3) were excluded, as it was uncertain whether these conditions might hamper the SPF analyses. These women were included in studies II-V.

- Studies I, II, III-IV and V differ in the number of patients included. This has been explained in the text and in the flow-charts. The differences were due to discrepancies in the numbers of analyses carried out in the study populations (SPF, serum estradiol and progesterone) the impossibility of retrieving paraffin blocks, as well as difficulty in staining biopsy samples in occasional cases.

- An analysis of clinical and demographic data, however, showed that the characteristics of the women were similar in all studies.

- In studies I and II different cut-off levels for SPF were used (14% and 12%). In these studies the best explanatory cut-off level was used, as there is no established cut-off level for SPF in cervical cancer.

RESULTS

General characteristics of the different study populations are presented in Table IV, and ten-year mortality rate by clinical stage are given in Table V. The overall ten-year mortality rate in the whole population was 42.1.6%. The ten-year mortality rate was 45.6 % in study I, 44.5 % in study II, 39.0 % in studies III-IV and 39,8 % in study V. Mean age for premenopausal women was 37.7 years, and 68.2 years for postmenopausal women in the whole study group of 190 patients. The ten-year mortality rate was 28% in pre- and 51% in postmenopausal women (p=0.002) in the whole study group. Stage specific mortality is given in Tables II and IV.

Table IV. General characteristics of the study populations in studies I, II, III-V and V.

	Study I n=103		Study II n=128		Studies III-IV n=128		Study V n=128	
Mean age (SD), years	56.4	(16.7)	55.5	(17.6)	59.7	(16.7)	59.7	(16.5)
Pregnancies (no., SD)	2.8	(2.0)	2.8	(1.9)	3.1	(2.1)	3.1	(2.1)
Parity (SD)	2.5	(1.8)	2.4	(1.7)	2.7	(1.8)	2.7	(1.9)
Postmenopausal (no., %)	64	(62.1)	78	(60.9	88	(68.9)	89	(69.5)
Menarche (mean age, SD), years	13.7	(1.7)	13.5	(1.6)	13.5	(1.7)	13.5	(1.6)
Menopause (mean age, SD),years	48.8	(4.1)	48.2	(4.9)	48.1	(4.8)	48.3	(4.8)
Body mass index (mean, SD)	24.9	(4.7)	25.0	(4.7)	25.1	(4.8)	25.2	(4.8)
Smokers (no., %)	32	(34.8)	53	(46.9)	46	(45.6)	48	(40.6)
Ever OC use (no., %)	36	(37.5)	48	(40.0)	42	(34.3)	46	(33.3)

Table V. Ten- year mortality rate by clinical stage in the patients of the different studies.

Clinical stage	Study I n=103			Study II n=128			Study III-V n=128			Study V n=128		
	Patients		Mortality rate	Patients		Mortality rate	Patients		Mortality rate	Patients		Mortality rate
	no.	%	%	no.	%	%	no.	%	%	no.	%	%
IB	37	35.9	16.2	53	41.4	17.0	54	42.1	22.2	52	40.6	21.1
IIA	14	13.6	50.0	18	14.1	38.9	14	10.9	42.9	15	11.7	40.0
IIB	18	17.5	55.6	18	14.1	50.0	18	14.0	33.3	19	14.8	36.8
IIIA	4	3.9	75.0	4	3.1	75.0	5	3.9	80.0	5	3.9	80.0
IIIB	25	24.3	68.0	29	22.7	75.9	30	23.3	63.3	30	23.3	63.3
IV	5	4.9	80.0	6	4.7	83.3	7	5.4	85.7	7	5.4	85.7

Study I

Of the 103 women, 64 (62. 1%) were post- and 39 (37.9%) premenopausal. Thirty-two (34.8%) of the women were current smokers. There was no difference in smoking habits between pre- and postmenopausal women. Oral contraceptives had been used by 36 (37.5%) of the women (for 2-243 months), while 3 (3.2%) women were receiving or had received hormone replacement therapy (HRT) (for 2-114 months). Clinical staging was similar to that in the whole study population. The cancer was diagnosed at an earlier stage in pre- (mean IIA) than in postmenopausal (mean IIB-IIIA) women (p=0.0001).

Mean se-P was 2.0 nmol/L (range 0.2-16.4) and mean se-E2 87.5 pmol/L (range 8.2-1165.0). The corresponding mean concentrations of se-P and se-E2 in pre- and postmenopausal women were 3.3 and 1.2 nmol/L, and 168 and 41 pmol/L respectively.

Ploidy ranged between 1.9 and 8.4 and the mean SPF was 16.3 (SD 7.5, range 0.0-35.7, median 15.3%). There were 48 peridiploid (47.1%) and 54 aneuploid (52.9%) tumors. There were no significant differences in mean SPF and the proportion of peridiploid/aneuploid tumors between pre- and postmenopausal women. A linear stepwise regression analysis of se-P concentration (steps: 0.1 nmol/l) and SPF (steps: 1%) showed a significant correlation between the two variables at cut-off values for se-P of 2.4-2.6 nmol/L and SPF of 14% (Table VI).

To study possible independent associations between SPF ≥14% and eight variables, that included ploidy-level, se-P and E2 concentration, clinical stage, histopatholoical grade, parity, menopausal status and smoking, a logistic regression analysis was performed. Aneuploidy (OR10.0, 95% CI 3.3-29.7), se-P (OR 7.5, 95% CI 1.4-40.8) and smoking (OR 3.0, 95 % CI 1.1-8.8) showed significant associations with SPF, while the other variables did not.

Table VI. Association between S-phase fraction and serum progesterone levels.

	Progesterone (nmol/L)	
S-phase fraction	<2.6 (%)	≥2.6 (%)
14%	45 (52.9)	16 (88.9)
<14%	40 (47.1	2 (11.1)

p=0.007 (Fisher's exact test)

Crude analyses were performed after dividing the study population into pre- (n=39) and postmenopausal (n=64) women. In fertile women the associations between SPF ≥14% and se-P 2.6 nmol/L (OR 7.7, 95 % CI 1.7-56.4), aneuploidy (OR 14.9, 95% CI 3.1-113.1) and smoking (OR 11.1, 95% CI 2.2-86.1) remained significant. In the postmenopausal group the significant association with aneuploidy (OR 0.9, 95% 2.0-18.8), but not with smoking (OR 11, 95% CI 0.3-3.1) remained. It was not possible to calculate the odds ratio for se-P ≥2.6, as there were only three such cases in this group of women, and all three had an SPF ≥ 14%.

Study II

The main purpose of this study was to evaluate the possible correlation of serum progesterone and estradiol with mortality. An advanced clinical stage and an S-phase fraction of 12% or above were significantly associated with reduced survival in those women who eventually died of their cervical cancer (Table VII). Among the 12 premenopausal women who died of their disease, an estradiol/progesterone ratio below 60 correlated to a shorter survival, as compared to those with a ratio above 60, and the difference almost reached statistical significance (p=0.08). The mean and median survival in these 12 women was 20.0 months and 10.5 months, respectively. Clinical stage and SPF ≥12% correlated to duration of survival in women who eventually died, while climacteric status, differentiation, smoking, BMI and aneuploidy did not.

Table VII. Association of survival months with clinical stage, SPF, and estradiol/progesterone ratio in women who died of cervical cancer.

	No. of patients	**Mean survival (months)**	**No. of patients**	**Mean survival (months)**	**p-value**
Stage IB-IIA *v.* IIB-IV	27	45.1	52	22.2	0.0001
S-phase ≥12% *v.* S-phase <12%	41	24.1	22	39.9	0.03
Serum estradiol/progesterone ratio <60 *v.* ≥60 (only premenopausal women)	7	11.9	5	31.4	0.08

When serum progesterone and estradiol were analyzed as continuous variables and survival curves were estimated by linear regression in the premenopausal women, a slight decrease in survival was found with increasing serum progesterone and an increase in survival with increasing serum estradiol. Both correlations were not significant. However, increasing estradiol/progesterone ratios significantly (p=0.02) correlated with increasing survival (Fig.11, below).

Forty-five postmenopausal women died of their disease. There was no tendency toward any association between survival and either serum estradiol, serum progesterone, or the estradiol/progesterone ratio.

Serum was on average sampled on day 10.8 of the menstrual cycle in the deceased premenopausal women. There was no association between months of survival and menstrual day at serum sampling (p=0.68). When survival time was dichotomized, women who died in less than 12 months after diagnosis had their serum sampled on menstrual day 10.4, as compared to 11.3 (p=0.83) in those who survived 12 months or more. When the cut-off level was set to 20 months, those who survived longer had their serum sampled at day 13.0 of the menstrual cycle compared to day 10.1 (p=0.54) in those with shorter survival.

Among all premenopausal women, the deceased women on average had their serum collected on day 13.0 of the menstrual cycle, as compared to day 14.4 (p=0.65) in survivors.

Premenopausal deceased women were dichotomized into stage IB-IIB (n=7) versus IIIA-IV (n=5). The mean estradiol/progesterone ratio was 104.3 in the former group, as compared to 60.7 in the latter (p=0. 47). There was no difference in estradiol/progesterone ratios and cancer stage in the whole material of premenopausal women.

A high S-phase fraction was associated with fewer months of survival. An S-phase fraction of 12 % or above was significantly associated a low estradiol/progesterone ratio (37.6 v. 185.1; p=0.02) but not with aneuploidy (83.3% v. 16.7%; p=0.06)).

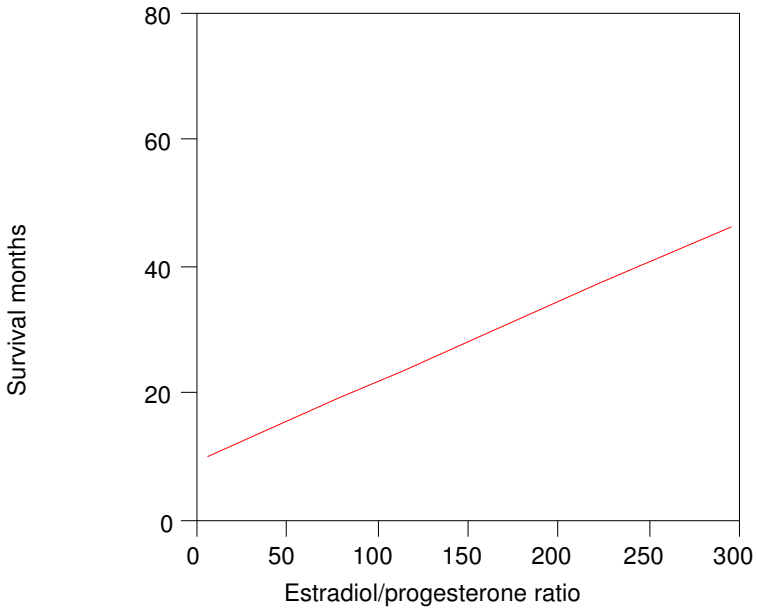

Figure 11. Correlation between serum estradiol/progesterone ratio and survival in 12 premenopausal women who died of invasive cervical cancer (p=0.02).

Study III

The aim of study III was to look for correlations between expressions of individual tumor markers, in particular combinations of tumor marker expression, and ten-year survival. Expression of three tumor markers significantly correlated to ten-year mortality after adjustment for cancer stage (IB-IIA v. IIB-IV), i.e., absent expression of p53 and high expression of c-myc or COX-2. CD4 expression correlated to decreased mortality, though not significantly (p=0.08), but was included in the analyses of combinations (Table VIII).

Table VIII. Ten- year survival rate and expression of tumor markers at cervical cancer diagnosis.

Expression	**Survival, cases %**	**Survival, comparison group %**	**OR**	**95% CI**	**p-value**
p53 >0% (n=77) *v.* 0% (n=50)	65.8	49.0	2.88	1.27–6.87	0.01
CD4 ≥20% (n=37) *v.* <20% (n=86)	70.3	53.5	2.28	0.94-5.86	0.08
c-myc ≥50% (n=47) *v.* <50% (n=79)	48.9	64.6	0.41	0.18-0.93	0.04
COX-2 intensity high (n=23) *v.* absent/low/moderate (n=103)	43.5	62.1	0.29	0.10-0.81	0.02

Ten combinations correlated significantly to overall ten-year survival after adjustment for stage. All four single markers were involved in these combinations. A high survival rate (76%) and odds ratio (3.73) were found with the combination of low expression of COX-2 and high expression of CD4+ or expression of p53 and high expression of CD4 (65%) odds ratio (3.56). A strong correlation to overall survival was also evident with the combination of any p53 expression and low expression of c-myc (Table IX). The differences in survival rates between patients with tumors with one of these combinations and the remaining study population varied between 19% and 35%.

Five of these combinations of biomarkers correlated inversely to survival rate. Of these five, four combinations included absence of p53 expression. The absolute differences in poor survival rates, as compared to the remaining study population, varied from 35% (high COX-2 intensity and c-myc expression) to 19% (high c-myc and absent p53 expression). The former combination correlated to very poor survival, but this group of women was small.

Table IX. Combinations of tumor markers related to prognosis in 128 women with invasive squamous cell cervical cancer.

	No. of cases	Ten-year survival		OR	95% CI	p-value
		Cases %	Comparison group %			
COX-2[1] **and** CD4+ ≥20%	33	75.8	53.3	3.73	1.42-11.0	0.01
p53 >0% **and/or** CD4+ ≥20%	93	64.5	45.7	3.56	1.43-9.44	0.008
p53 >0% **and/or** c-myc <50%	111	62.2	41.2	3.36	1.09-10.93	0.04
COX-2[1] **and** p53 >0%	59	69.5	50.7	3.09	1.36-7.45	0.009
c-myc <50% **and/or** CD4+ ≥20%	88	64.8	46.2	2.76	1.18-.67	0.02
p53 =0%**and/or** c-myc ≥50%	81	51.8	71.1	0.32	0.12-0.75	0.02
COX-2[2] **and/or** p53 =0%	67	47.8	71.2	0.25	0.10-0.37	0.0002
p53 =0% **and** CD4+ <20%	33	42.4	64.5	0.29	0.11-0.73	0.01
p53 =0% **and** c-myc ≥50%	16	37.5	62.4	0.25	0.07-0.79	0.02
COX-2[2] **and** c-myc ≥50%	11	27.3	62.3	0.13	0.02-0.52	0.007

[1]Absent, light or moderate staining intensity. [2]High intensity.

The four single markers and the ten combinations were finally analyzed for temporal survival trend using Cox regression. None of the single markers remained significantly correlated to survival. Five combinations correlated significantly to high or poor survival after adjustment for clinical stage. These were the combinations of absent COX-2 and expression of p53 (p=0.04, survival 70% v. 51%) and four combinations indicating poor prognosis, i.e., absence of p53 and/or c-myc expression (p=0.04, 52% v. 71%), COX-2 expression and/or absence of p53 expression (p=0.03, 48% v. 71%), absence of p53 and/or c-myc expression (p=0.04, 52% v. 71%), and COX-2 and c-myc expression (p=0.04, 27% v. 62%). Three combinations showed borderline significance (p=0.05).

Study IV

The aim of this study was to find any correlations between serum progesterone, serum estradiol and smoking, on the one hand, and expression of tumor markers, on the other, in an attempt to find biological evidence underlying these risk factors for cervical cancer.

Low expression of EGFR was significantly associated with high serum estradiol (195 pmol/L v. 55 pmol/L, p=0.0007).

High progesterone levels were associated with high c-myc expression (2.7 nmol/L v. 1.3 nmol/L, p=0.01), and inversely with p53 expression (1.4 nmol/L v. 2.5 nmol/L, p=0.046). In fertile women, serum hormones with high and low c-myc expression were sampled on menstrual cycle days 11.6 and 12.8, respectively (p=0.78). The corresponding values for p53 were on days 11.4 and 14.0 (p=0.35). The associations with hormones were relatively higher in premenopausal women. Thus, serum progesterone was increased more than two-fold (4.63 nmol/L v. 1.87 nmol/L; p=0.11) when 50% or more of the tumor cells were c-myc stained, and when there was no p53 expression (4.73 nmol/L v. 2.08 nmol/L; p=0.10), as compared to the findings in the remaining study population. The absence of significant p-values might have been due to the small number of available fertile women (n=23). Expression of the tumor markers was not associated with age.

Current smoking was inversely correlated to p53 expression (34% v. 61%, p=0.008). History of oral contraceptive use was not associated with expression of any tumor marker.

Study V

LRIG1 was expressed in various subcellular locations: nuclear, perinuclear and cytoplasmic compartments. It was dichotomized into no expression/expression.

In the whole study population LRIG1 was not expressed in 67 (52.3%) women, lightly expressed in 46 (35.9%), moderately expressed in 13 (10.2%), and highly expressed in 2 (1.6%) women. There was a tendency toward lower expression in more advanced stages, but the difference was only of borderline significance. When stage was dichotomized into IB-IIA versus IIB-IV, however, the difference in expression between early-stage cancer (56.7%) and late-stage cancer (37.7%) was significant (p=0.03).

Women with cancer stage IB and LRIG1 expression had a significantly better prognosis than those in whom LRIG1 was absent, and the odds ratio was high (90% v. 64% survival, p=0.02; OR 5.1, 95% CI 1.3-26.4). The differences in survival rates in relation to LRIG1 expression was not significant in women with other stages, but there was a relative difference among women with stage IIA that was equal to that in women with stage IB (75% v. 43% survival, p=0.20). This was not significant but might have been due to the small study population (n=15) in stage IIA. There was a tendency for expression of LRIG1 to be progressively less important for survival with advancing stage. The survival curve (Kaplan Meier) in Figure 12 is given for stages IB/IIA. On crude analysis the survival rate for stages IB and IIA combined was 86.8% in cases where the tumor expressed LRIG1, compared to 58.6% in cases when LRIG1 expression was absent (p=0.008; not shown in the article).

LRIG1 expression did not correlate to expression of any of the other tumor markers investigated. The kappa index was invariably close to zero, where 1.0 stands for total agreement

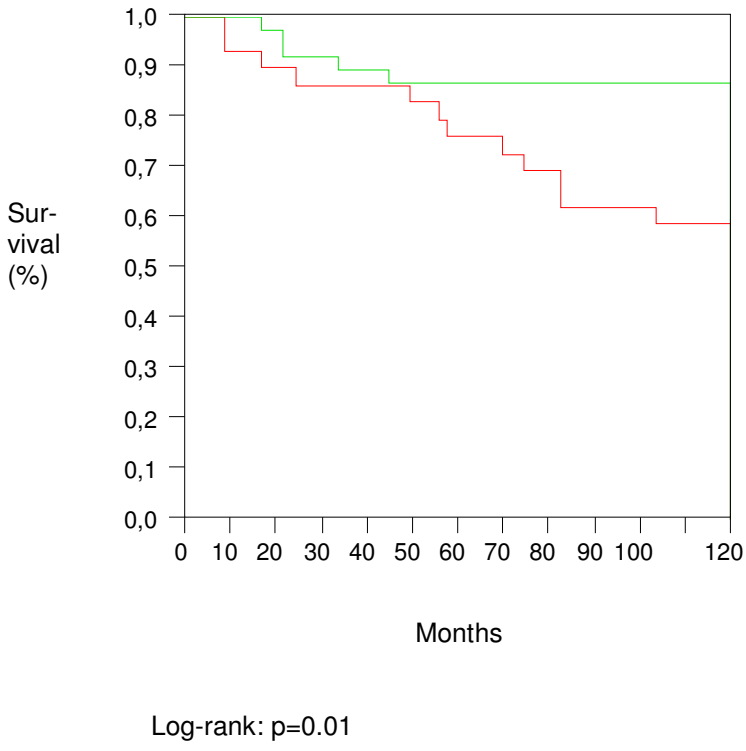

Figure 12. Survival from squamous epithelial cervical cancer in women with clinical stage IB/IIA in tumors with (green) and without (red) LRIG1 expression.

Finally, serum estradiol and progesterone levels, and smoking habits, were compared with expression of LRIG1. Premenopausal women lacking LRIG1 expression had significantly higher progesterone levels than women with tumors expressing LRIG1 (6.02 nmol/L v. 1.79 nmol/L, p=0.03) and the smoking frequency was significantly increased in these women as compared to those with LRIG1 expression (67% v. 25%, p=0.04). No such differences were found in postmenopausal women.

DISCUSSION

Cervical carcinoma remains an important health problem in both developed and developing countries, even though population-based screening programs are widely available. The age-specific incidence increases from the age of 30, thus affecting fertility and childbearing. Only a very small proportion of invasive cancers that are diagnosed in the earliest stages are available for fertility-preserving surgery. Treatment with radiation and chemotherapy affects the ovaries and causes premature ovarian failure with concomitant infertility and premature menopause.

The major results of this study were the findings that prognosis of cervical cancer correlated with serum sex steroid hormone levels, expression of LRIG1 as well as combinations of tumor markers, and that there were reciprocal correlations between these factors, including smoking. The findings are generally novel, but if confirmed they would increase our knowledge about tumor biology, tumor behavior, and prognostic prediction in cervical cancer.

Progesterone

There was a significant positive correlation between a low serum estradiol/progesterone ratio and short survival in premenopausal women who eventually died of cancer ($p=0.02$) (study II). Caution must be observed when interpreting these results, as the number of women was small. We also found correlation between absent LRIG1 expression and increased serum progesterone levels in the premenopausal group of women (study V). In the whole study population, there were additional correlations between sex steroid hormone levels and specific tumor markers and these will be discussed in connection with each tumor marker (study IV).

Progesterone has been indicated as the major candidate for a role in cervical neoplasia because of its immunosuppressive effect and for its possible connection with HPV infection (186). Cell transformation in HPV transfected cervical cells has been reported to occur when progesterone and ras oncogene, or oral contraceptive gestagens, have been added (52). Higher serum progesterone levels after adjustment for menstrual phase have been reported to be correlated to higher prevalence of HPV infection (187).

We found an increase in cell proliferation, measured as the number of cells in the S-phase, with high serum progesterone levels in invasive cervical cancer (study I).

There has been little biological evidence *in vivo* for a role of oral contraceptives and female sex steroid hormones in cervical cancer. In an experimental study, enhanced colony-forming efficiency was found in the HPV 16-DNA-integrated cervical cancer cell line, CaSki, after at least three days of progesterone treatment (53). The progesterone antagonist RU 486 was able to abrogate the enhancement of progesterone on cell growth. Progesterone and glucocorticoid hormones increased HPV mRNA and significantly stimulated viral replication (51, 53, 188).

There is high expression of both progesterone and estrogen receptors in normal epithelium of the transformation zone compared to ectocervical epithelium in women of fertile ages (189). It is known that estrogen but not progesterone receptors are successively lost with progression of cervical neoplasia, but the possible clinical relevance of this loss is not known (190). HPV 16 and HPV 18 contain progesterone and glucocorticoid response elements that will increase expression of the HPV E6 and E7 oncogenes and facilitate immortalization with gestagenic stimuli. Increased transcription of E6 and E7 is crucial in cell transformation, and a relation to activated ras oncogene has been reported (51, 53, 55, 191, 192). These previous experimental findings support our results of a shorter survival in premenopausal women who have a low estradiol-progesterone ratio (study II).

Estrogens

There is also evidence that estrogens play a role in the malignant progression of cervical carcinoma. Serum estrone was found to be higher in patients with CIN who were HPV positive than in HPV negative women with or without CIN (193). Steroid sex hormone levels in women have been evaluated as a potential aid in grading cervical neoplasia, but were not found to be useful (194). These results might support our clinical findings of a positive effect of estrogen (high E2/P-ratio) on months of survival and with the report that estrogens reduce susceptibility to primary HPV infection (195). The reported 'estrogens' are not estradiol, however, as in the present study, but several synthetic compounds with estrogenic effects as in oral contraceptives. There is also a possibility that estrogens are involved in different processes during acquirement of HPV infection and at different stages of carcinogenesis, although this is speculative. Moreover, results from *in vitro* experiments may differ from those in clinical studies.

Estradiol/progesterone ratio

As discussed above, there was a significant positive correlation between a low serum estradiol/progesterone ratio and shortened survival in those premenopausal women who died of cancer (study II). This finding indicates that there is in fact a clinical association between sexual steroid hormones, in this study endogenous estradiol and progesterone, and survival months in premenopausal women who died of invasive squamous cell cervical cancer, providing new evidence for a role of hormones in cervical cancer.

There is previous epidemiological and experimental evidence, as discussed above, that progestogens and estrogens may play a role in cervical carcinogenesis (195). There are several possible reasons for refuting these reports. Epidemiological studies may lack control for all possible confounding factors, and important subgroups may not have been analyzed.

Positive laboratory results in animal studies might be species-specific. Concentrations of sex hormones added in laboratory studies on human cervical tissue, normal or cancerous, might not be equivalent to the hormone concentrations found *in vivo*.

Not much work has been done regarding prognosis of SCC in relation to endogenous sex steroid hormones. The present study has provided clinical evidence that estrogen and progesterone may have a role in prognosis. When the present laboratory results are summarized, it is evident that our finding of shorter survival in women with a low estradiol/progesterone ratio, as compared to those with a high ratio, is biologically plausible in view of the negative role of progesterone. The possibly positive effect of estradiol found in this study needs to be confirmed.

Oral contraceptives

The role of OC in cervical neoplasia is still not completely established. An increased risk of CIN with long term use of OC has been found in most studies. To our knowledge, no attempt has been made to correlate risk of CC with hormonal balance in combined OC. Women with CC usually become infertile due to treatment and do not receive OC. Modern treatment with fertility saving surgery in early stages of cancer will give women a need for contraceptives. The role of OC for prognosis in CC is not known.

HRT

Cervical cancer risk has not been shown to be affected by HRT (196, 197). As for OC the role of HRT (estrogen only, or estrogen combined with gestagen) for prognosis in CC is not known. According to a study by Lauritzen HRT is possible without drawbacks following treated CC (198). In our study only five post-menopausal women had been on HRT, the number is too small to provide any results.

Smoking

The present study included evaluation of pre-treatment cancer biopsy samples in relation to smoking habits. An increased tumor growth (SPF $\geq$ 14 %), was associated with smoking (study I). Highly significant associations were found between absence of p53 staining and smoking ($p=0.008$) (study IV). Smoking also correlated to absent LRIG1 expression (study V). Few previous studies have addressed correlations between tumor marker expression and smoking, and then only with reference to single markers. In one study heavy smoking was found to be positively associated with Ki-67 staining (199). The fragile histidine triad (FHIT) gene and its protein, a tumor suppressor, has been studied in cervical cancer, initially through FHIT-specific DNA sequences. Loss of the FHIT gene was significantly associated with smoking in women with cervical cancer (200). Another study indicated that the FHIT gene may be altered in smoking-associated cervical cancers (201). Finally, the p16 protein has been investigated in relation to smoking in cervical cancer. This protein controls cell growth by preventing inactivation of retinoblastoma protein, a protein which is degraded by the E7 oncogene. Aberrant p16 methylation,

which was associated with loss of p16 protein, differed markedly between smokers and non-smokers with invasive cancer, with considerable differences in CIN, but non-significant differences in normal tissue (202). In one study smoking predicted worse overall survival in women with locally advanced cervical carcinoma treated with chemoradiation (203).

S-phase fraction

Serum progesterone ≥ 2.6 nmol/L, smoking, and aneuploidy were significantly associated with proliferation, i.e., SPF ≥ 14 %, after adjustment for all factors included in the study. The associations with serum progesterone and smoking were only found in premenopausal women (study I). In both pre- and postmenopausal women who died of cervical cancer, an S-phase fraction of 12% or above correlated with reduced months of survival (study II).

A mean shorter survival in women with a high S-phase fraction is compatible with the finding in the present study of a correlation between high serum progesterone and a high S-phase fraction. Since this study was carried out a number of studies have assessed the value of SPF in providing prognostic information, in the choice of treatment and in monitoring the treatment effect, but the results have been conflicting. The cut-off values used for SPF varied between 7% and 20%. In six studies SPF measurement was found to be clinically useful, in particular for prognosis, while four studies reported negative results (142, 204-212).

There are several possible reasons for the discrepancies between these studies. The S-phase fraction is difficult to measure. In one study SPF could be evaluated in only 22% of aneuploid tumors (142). Experience and laboratory methods are of great importance. In addition, different end points have been used, such as presence of lymph node metastasis, recurrences, or overall survival. In addition, many studies have focused on specific tumor stages which make comparisons between studies difficult. Our study showed no effect of SPF on overall survival, but a high SPF was associated with fewer survival months in those patients who eventually died. In patients who are not radically cured, the tumor proliferation rate might influence overall survival to a limited extent.

Ploidy

Ploidy has not shown to be a useful prognostic factor in cervical cancer (142). In this study aneuploidy showed correlation to a high SPF but not to survival (studies I and II).

Tumor markers

p53

In multivariate analysis adjusting for clinical stage, p53 expression correlated significantly to a favorable outcome, but this was not true with Cox regression analyses. There was a weak, but significant association between the absence of p53 expression and high serum progesterone (p=0.046). The findings indicate that p53 act as a tumor suppressor in cervical cancer.

The p53 tumor suppressor gene encodes the p53 protein. It is activated in response to DNA damage and p53 protein causes cell cycle arrest by blocking the cell at the G0/G1 phase prior to DNA replication, and thereby aids the DNA repair process and prevents mutations (100). The HPV E6 oncogenes bind p53 and direct its rapid degradation, an important step in viral DNA replication. Inactivation of p53 by mutations is less common in cervical cancer (213). In a recent study that only included early-stage cancer, p53 expression gave no prognostic information (214). In the present study, however, combinations of tumor markers that included p53 expression still correlated to prognosis after adjustment for stage (study III).

c-myc

Low expression of c-myc correlated significantly with survival in multivariate analyses, but not with use of Cox regression. Highly significant associations were found between strong c-myc staining (≥50%) and increased serum progesterone (p=0.01) (study III). c-myc is one of the 'classic' oncogenes, and it binds to hundreds of potential target genes and contributes to increased proliferation, loss of differentiation, immune suppression, neoangiogenesis, and apoptosis (215). A major function is activation of transcription. It has also been shown that the HPV E6 gene increases telomerase expression and TERT gene activation (telomerase reverse transcriptase), which is crucial for immortalization, through induction of c-myc (216). c-myc expression has been found in many studies to be associated with poor prognosis, but other studies have shown no association between c-myc and prognosis (98, 99, 217).

COX-2

Low expression of COX-2 correlated significantly to survival in this study after adjustment for stage, but not when Cox regression was used (study III). COX-2 responds to a variety of mitogenic and inflammatory stimuli and is thought to be involved in a number of steps in cancer development in itself or through prostaglandins. Increased expression correlated to the presence of HPV E6 and E7 proteins in one study (218). COX-2 expression is associated with a number of events such as proliferation, inflammatory response to tumors, tumor invasion and angiogenesis, and lymph node metastases (126). It has recently been suggested that one mechanism is the up-regulation of the HPV oncoprotein E5 (219).

COX-2 expression has been evaluated with different diagnostic criteria in cervical cancer. In one study a high tumor/stroma COX-2 ratio was found to be independently correlated to a lower overall survival rate (220). In other studies specific clinical stages, COX-2 positivity as such, and intensity of COX-2 expression have been evaluated (221, 222). COX-2 expression has also been reported to correlate with recurrence of positive para-aortic lymph nodes and distant metastases, but not with prognosis (223, 224). Expression of COX-2 and VEGF has also been reported to be higher in younger than in older women with advanced cancer (127). In the present study a number of combinations of tumor markers that included COX-2 expression correlated to survival rate. Sex steroid hormone levels or smoking were not associated with COX-2 expression.

CD4+

There is a complex relation between different factors that are related to immune response to tumors, among them CD4+ and CD8+ T-cells; CTL effector cells, derived from CD8+ T cells; B lymphocytes; and T- helper 1 and T- helper 2 responses. Successful immunity to cancer will require activation of tumor-specific CD4+ and CD8+ cells and has provided a basis for recent tumor immunotherapy strategies (225, 226).

CD4+ T-helper cells play a crucial role in the cell-mediated immune response, e.g. toward HPV-infected cervical cells (227). The presence of a large number of NK cells, and increased serum CD4+ and CD8+ T-cell levels, have been associated with a favorable response in patients with cervical cancer and also when only early-stage cancer was included (123, 228). An immunological response to HPV epitopes correlated to prognosis in one study (229).

Sex steroids seem to play a role in the immune response, with a positive effect of estrogens and a negative effect of progestogens (230). Little is known about these effects in the cervix, but it is well known that immunocompromised women, such as in HIV infection, have a high incidence of CC (27).

In the uterine cervix perhaps the most studied immune response factor is the Langerhans cells. Langerhans cells present antigen for the T cells and are found in low levels in the uterine cervix of smokers as compared to non-smokers (231-233).

In our study CD4+ was associated with increased survival, however non-significantly ($p=0.09$) (study III).

LRIG1

In the present study LRIG1 appeared to be a significant prognostic predictor in early-stage cervical cancer, with similarity to tumor suppressors. LRIG1 expression was independent of that of the other investigated tumor markers. If this can be confirmed, LRIG1 expression might be of clinical importance. In clinical stage IB 58% of the tumors showed LRIG1 expression, but there was a decline with increasing stage. In a previous study no staining of normal epithelium was observed (110). Ninety per cent of women with stage IB cancer and LRIG1 positivity survived, as compared to 64% of women without LRIG1 expression ($p=0.02$). LRIG1 expression did not predict the prognosis in advanced stages, but there was a marked though non-significant, difference in stage IIA between women who survived and those who died (study V).

Other tumor markers, e.g. TGF- ß, have also shown discrepancy in the results in different clinical stages. In breast cancer, mouse models have indicated that TGF-ß has biphasic effects on tumor pregression, acting as a tumor suppressor in early stages of cancer and promoting invasion and metastasis in later stages (234).

LRIG1 has recently been investigated as a prognostic predictor in 38 cases of squamous cell carcinoma of the skin (235). In skin cancer, differentiation is an important prognostic variable. High LRIG1 expression correlated to well-differentiated tumors, while low expression correlated to low differentiation. A significantly increased survival rate with high expression, of the same magnitude as in the present study, was reported, providing further evidence that LRIG1 acts as a tumor suppressor.

Previous findings of an inverse relationship between LRIG1 and EGFR expression could not be confirmed in the present study (236-238). EGFR was expressed in the large majority (93%) of tumors, but even when different cut-off values for the frequency of expression were analyzed, we failed to demonstrate any correlations. Gene amplification with fluorescence in situ hybridization (FISH) might be a diagnostic alternative (239).

High serum progesterone levels and smoking correlated to absent LRIG1 expression in pre- but not postmenopausal women. This fits with our finding that both high serum progesterone levels and smoking correlated to a high S-phase fraction and that low serum progesterone combined with high estradiol levels (E_2/P ratio) correlated to a longer duration of survival in fertile women (studies I and II). These observations provide further evidence that progesterone and smoking exert negative effects in cervical cancer.

The difference in results between pre-and postmenopausal women might seem contradictory. However, serum levels of sex steroids are minimal and stable after the menopause. The difference in LRIG1 expression between smokers and non-smokers was, like that in progesterone levels, pronounced. We have no explanation for the discrepancy between pre- and postmenopausal women with regard to smoking habits.

EGFR

EGFR, a glycoprotein associated with proliferation and located at the cell surface, is over-expressed in a wide variety of cancers. It has been found to be associated with a poor prognosis in a number of cancer types and is one of the most evaluated prognostic markers for cervical cancer, but with conflicting results. In this study EGFR expression had provided no prognostic information, which was also the result of another study (81). In yet other studies, EGFR expression correlated to a poor prognosis, also in multifactorial analyses, but not in a recent study, which also included p53 (83, 86, 240). In another recent report it was concluded that simultaneous expression of EGFR and HER 2 (c-erbB-2 /neu) correlated to a worse prognosis (241). EGFR expression has also been found to correlate to a favorable prognosis in multivariate analyses (242).

Low EGFR staining and high serum estradiol, however, correlated strongly in our study, a novel and unexpected observation that is not easily explained (study III). The estrogen-induced proliferation of the endometrium is well known, but the question whether estrogens have the reverse effect on cervical squamous epithelium has been less studied.

Ki-67/MIB-1

Ki-67 expression did not correlate to prognosis in the present study (study III). In a number of studies on cervical neoplasia, Ki-67 expression has been found to be increased with advanced stage and has been used to predict the prognosis of CIN (243). In one study MIB-1 was an independent prognostic factor in early stage cervical carcinomas (93). Other studies have not shown MIB-1 expression to be clinically useful.

p27

p27 is a wild type (normal) p53 inducible protein that blocks cell cycle proliferation in the G1-S phase. In several studies it has been concluded that expression of p27 is not clinically useful in cervical cancer, and the same conclusion was drawn in the present study (study III) (244, 245). Studies in which expression correlates to survival are probably confounded by differences in expression in different clinical stages (246).

E-cadherin

Among several proteins responsible for intercellular adhesion, one of the most studied is E-cadherin. E-cadherin has been shown to decrease from normal cervical epithelium, through CIN, to invasive cervical cancer (247). Increased cytoplasmic staining has been observed with worsening differentiation in invasive cervical carcinomas and a strong correlation to lymph node metastasis was found in a recent study (117, 248). It has been reported that silencing the oncoprotein E7 restores functional E-cadherin expression *in vitro* (249). In the present study E-cadherin expression appearad to have no clinical role (study III).

CD44

CD44 consists of a heterogeneous family of cell-surface glycoproteins that are involved in cell adhesion. Most widely studied in cervical cancer is CD44. In addition to standard CD (CDs) its exon CD44v6 (splice variant) has received most attention. In CIN a reduced expression of both CD44s and CD44v6 has been found as compared to the expression in normal epithelium in one study, but not in another, and was found to further decrease in invasive cancer (250-252). In invasive cervical cancer CD44s and particularly its isoform CD44v6 have been reported to strongly and independently correlate with a poor prognosis (120, 121). Invasive cervical cancers that are CD44v6 positive have been reported to respond better to chemotherapy than those that are negative (253). In the present study no role was found for CD44 in cervical cancer (study III), but in retrospect it might have been more useful to include CD44v6.

VEGF

Vascular endothelial growth factor (VEGF) is a cytokine. It is the major protein that induces angiogenesis, and is the most frequently studied marker. Angiogenesis is an early event in cervical neoplasia and VEGF expression is increased in invasive cervical cancer as compared to CIN III (125, 254). VEGF expression has been reported to correlate to poor prognosis in many, but not all studies of cervical cancer (113, 114, 255). In the present study no correlation to prognosis was seen (study III). The increased expression and the correlation to a poor prognosis in many studies might reflect an advanced stage of cancer as such. Serum VEGF was measured in patients with invasive cervical cancer and correlated to advanced and large tumors (256). A recent study indicated that serum VEGF may be a useful prognostic factor in CC (257).

Telomerase

Telomeres are repetitive DNA sequences at the ends of chromosomes that are shortened at each cell division. Progressive shortening of DNA may cause chromosome instability and cell death. Telomerase is able to synthesize repetitive sequences onto chromosomal ends maintaining telomere length, thus contributing to immortalization of cancer cells (258). We included telomerase in our study but the IHC staining failed at our laboratory and again at one of the most experienced laboratories in Sweden, the Human Proteome Resource Laboratory, Uppsala.

Combinations of tumor markers and prognosis

Specific genes regulate the proliferation of cells through stimulatory and inhibitory signals, and defects in these signal pathways are a characteristic in cancer. It is of importance to find new biological tumor markers that predict the outcome in each individual patient in order to apply earlier individualized therapy. Cancer treatment will increasingly be based on individualized therapy selected by specific diagnostic methods. If the increasing number of new promising targeted therapies – theranostics - will hold its premises, prognostic/predictive markers will become even more necessary in the future to select patients for individual targeted therapies.

Evaluation of the prognosis is of great importance in many situations, such as in the planning of treatment, and for providing the patient with information and advice. It seems unlikely that a single tumor marker will be the golden standard in prognostic prediction. This will necessitate evaluation of prognostic markers in cervical cancer by choosing a panel of markers with different modes of action. One purpose of this study was to improve prognostic prediction and find the most promising combination of prognostic markers relevant in cervical cancer and representing different mechanisms in carcinogenesis.

p53 expression and low expression of c-myc and COX-2 correlated significantly with ten-year survival. In addition, CD4+ expression was included in the analyses of combinations. When these four tumor markers were combined, two-by-two, ten combinations correlated significantly to ten-year survival. These included low COX-2 with high CD4+ expression (76% survival v. 53% in the remaining women; OR 3.73), and absent p53 with high COX-2 (48 % survival v. 71%; OR 0.25), while the corresponding figures for the combination of high COX-2 intensity and expression of c-myc were 27% v. 62% (OR 0.13).

None of the single markers correlated significantly with outcome in the final Cox regression analyses, while five combinations did. These included COX-2, c-myc and p53, but no combination included CD4+.

When this study was published there had been few attempts to evaluate prognostic prediction with combinations of tumor markers. There were no systematic studies, and those available included the two or three tumor markers (259, 260). Calculations of sensitivity and specificity were not attempted in this study, and were in general not included in other studies at the time of its publication. There is evidence in this study, however, that the sensitivity of many combinations is acceptable, while the specificity is expected to be quite low.

As none of the investigated single tumor markers was clinically useful when analyzed with Cox regression, the present study could serve as an example for future studies in the search for even better combinations of tumor markers.

CONCLUSION

This study has provided new possible factors for prognostic prediction in squamous cell cervical cancer ie., serum progesterone and estradiol, smoking, and combinations of tumor markers.

- Smoking and elevated serum progesterone levels correlated to increased tumor growth, measured as SPF, in premenopausal women.

- Low serum estradiol (E2) and increased serum progesterone (P) correlated to shorter survival in premenopausal women who died of cervical cancer.

- In logistic regression analyses p53 expression correlated to a favorable prognosis, while c-myc and COX-2 expression correlated to a poor outcome.

- Combinations of these tumor markers that also included CD4+ expression strengthened prognostic prediction expressed in terms of odds ratios.

- When tumor markers were included in Cox regression analyses no single tumor marker emerged as a significant prognostic factor, while certain combinations did.

- LRIG1 expression was evaluated for the first time in cervical cancer and was found to act as a tumor suppressor in early-stage cancer.

- Smoking emerged as a factor correlated to negative biological events in cervical cancer. Thus, smoking correlated to high SPF, short survival, absent p53 expression and absent LRIG1 expression.

- Serum progesterone also emerged as a factor correlated to negative biological events in cervical cancer. Thus, high serum progesterone correlated to high SPF, short survival when measured as E2/P, and with an unfavorable pattern of tumor marker expressions, i.e., low LRIG1, strong c-myc and low p53 expression.

FUTURE DIRECTIONS

- A further search for combinations of other tumor markers is warranted and necessary to strengthen prognostic prediction as described in this study.

- Novel markers need to be identified for screening, early diagnosis and prognosis, possibly by combining genomics and proteomics. This could be possible through development in molecular fingerprints both on the human genome (HUGO) and proteome (HUPO).

- Further studies of tissue markers to enable targeted therapy following development of "designed drugs".

- Consideration of tumor markers in evaluation of new treatments.

- Search for tumor markers that could be useful for monitoring of treatment, especially effect of treatment and early identification of recurrence.

- Further research on hormonal influence on the prognosis is required.

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