Associations between Ten Biological Tumor Markers in Squamous Cell Cervical Cancer and Serum Estradiol, Serum Progesterone and Smoking

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Abstract. *Background and Aim: To study possible associations between selected tumor markers and co-factors in squamous cell cervical cancer. Materials and Methods: Ten biological tumor markers representing different functions in carcinogenesis were diagnosed in 128 cases of squamous cell cervical cancer. These were p53, c-myc, EGFR, COX-2, CD4+, VEGF, E-cadherin, CD44, Ki-67 (MIB-1), and p27. Smoking habits and previous oral contraceptive use were registered. Serum estradiol and progesterone levels were evaluated in 80 women. Each marker was compared to these four variables. Results: Highly significant associaions were found between strong c-myc staining (≥50%) and increased serum progesterone (p=0.01), a low EGFR staining (<20%) and high serum estradiol (p=0.0007), and an absence of p53 staining and smoking (p=0.008). There was a association between the absence of p53 and high serum progesterone (p=0.046). Conclusion: The study supports a role of progesterone as a promoter of cervical cancer and indicates that smoking is associated with tumor development.*

Cervical human papillomavirus (HPV) infection is established as the main etiological agent for cervical neoplasia. HPV infection is commonly referred to as a necessary but not sufficient factor for invasive cervical cancer. Immortalization of the cervical cell is necessary for progress of cervical intraepithelial neoplasia (CIN) to invasive cancer. Integration of viral DNA to the host genome thereby enabling expression of viral oncogenes E6 and E7 seems to be a necessary step in immortalization and probably does not occur without the presence of co-factors (1). Smoking and oral contraceptive use have been the most widely studied epidemiological co-factors in cervical neoplasia (2, 3), while the role of endogenous sex hormones has not been established (4, 5).

There is little biological evidence *in vivo* for a role of oral contraceptives and female sex steroid hormones in cervical cancer. HPV has a tendency to transfect cells with progesterone receptors. Both HPV 16 and HPV 18 contain progesterone and glucocorticoid response elements that increase expression of the HPV E6 and E7 oncogenes, considered crucial in cell transformation, with gestagenic stimuli (6). Such a transformation has been reported to take place when progesterone or oral contraceptive gestagens were added (7). It is not known if this occurs *in vivo*. In an experimental study, an 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 (8). 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 (9).

In the epithelium of the transformation zone, where cervical neoplasia is initiated, 16-α-hydroxylation of estradiol occurs resulting in 16-α-hydroxyestrone, which is linked to malignant transformation of estrogen-sensitive cells transfected by HPV. When transgenic mice expressing HPV 16 were treated with estrogens, squamous cell carcinomas developed exclusively in the transformation zone (10, 11). In contrast, once invasive cancer has been established high serum estrogen levels might have a positive effect on outcome, while high serum progesterone levels could have a deleterious effect (5).

The first biological evidence for an etiological role of smoking in cervical neoplasms was the finding that levels of

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Table I. *Tumor markers included in the study and their major functions.*

Biological marker	Functions	Localization	Clone	Dilution	Reaction time (min)	Source	Antigene retrieval solution[1]
EGFR	Proliferation	Membrane	E30	1:100	30 min	DakoCytomation	TED pH 9 DAKO
Ki-67 (MIB-1)	Proliferation	Nucleus	MIB-1	1:100	30 min	DakoCytomation	TRS pH 6 DAKO
C-myc	Cell cycle progression, malignant transformation	Nucleus	9E11	1:100	30 min	Novocastra	TED pH 9 DAKO
p53	Cell cycle arrest, apoptosis DNA repair	Nucleus	DO-7	1:200	30 min	DakoCytomation	TED pH 9 DAKO
p27	Cell cycle arrest	Nucleus	SX53G8	1:50	30 min	DakoCytomation	TRS pH 6 DAKO
E-cadherin	Cell-cell adhesion	Membrane	NHC-38	1:25	30 min	DakoCytomation	TED pH 9 DAKO
CD44	Cell-cell adhesion	Membrane	DF 1485	1:50	30 min	DakoCytomation	TRS pH 6 DAKO
VEGF	Angiogenesis	Membrane	polyclonal	1:50	30 min	Santa Cruz Biotechnology	TRS pH 6 DAKO
Cyclooxygenase-2	Inflammation, angiogenesis, reduced apoptosis	Cytoplasm	SP 21	1:20	30 min	NeoMarkers	TED pH 9 DAKO
CD4+	Immune response	Intercellular	4B 12	1:50	30 min	Novocastra	TED pH 9 DAKO

[1]Antigen retrieval was carried out for 45 minutes in 96˚C water-bath; EGFR: epithelial growth factor receptor; VEGF: vascular endothelial growth factor; TED: DAKO TED pH 9 S2367; TRS: DAKO TRS pH 6.

nicotine, and its major metabolite cotinine, were increased forty-fold and four-fold, respectively, in the cervical mucus of women with CIN as compared to serum levels (12). Later, benzo(a)pyrene and tobacco-specific nitrosamines were identified in the cervical mucus of smokers but not of non-smokers (13, 14).

Little is known about what biological functions smoking and female sex steroids are correlated to in cervical cancer. The purpose of the present study was to select tumor markers that are known to be, or may be involved in cervical cancer. They were chosen to represent different steps in carcinogenesis and their expression was compared with smoking status and serum estradiol and progesterone levels.

Materials and Methods

The study population consisted of 128 women with invasive squamous cell epithelial cervical cancer stage IB to IV who were admitted to the Department of Gynecologic Oncology, Norrlands University Hospital, Umeå during 1984 to 1990. Clinical staging was made according to FIGO (15). The women were followed-up for at least ten years.

In addition to clinical history including smoking habits and oral contraceptive use, pretreatment serum estradiol and progesterone levels were evaluated. Estradiol and progesterone were evaluated by routine radioimmunoassay methods. Smoking was defined as daily consumption of cigarettes. Ex-smokers were excluded in the analyses.

The material was prospectively collected, but not consecutive. This was due to a period of absence of the initiator of the project (US) in the middle of the study, when serum hormones were not analyzed. Thus, in total 80 women had hormone analyses. The analyses were performed collectively at the end of the study. The treatment of choice was radiotherapy or radiation surgery in accordance with contemporary routines.

Ten tumor markers were chosen (Table I). 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.

Three-micrometer sections of the original paraffin blocks were reviewed by one of the authors (TT) and the most representative area(s) marked for tissue micro array (TMA).

Three-millimeter punch biopsies were taken from the biopsies and joined into TMA paraffin blocks, containing an average of 25 punch biopsies. Each TMA block also included two controls from human tissues, as specified by the producer.

Immunohistochemistry was carried out at the Department of Pathology and Clinical Cytology, Falun Hospital, as described elsewhere (16). In brief, 3-μm-thick sections from the paraffin blocks were cut and rehydrated. Immunohistochemical staining was carried out with the Dako Autostainer. Antigen retrieval was performed for all primary antibodies: overnight incubation in 0.1 citric acid, pH 7.2, at 65˚C. The Dako system uses biotinylated secondary goat antimouse antibody for the detection system and streptavidin-horseradish peroxidase conjugate for visualization of diaminobenzidine (DAB) solution. Endogenous biotin activity was blocked with a solution of streptavidin. The slides were weakly counterstained with hematoxylin and were mounted routinely. Details of the 10 antibodies chosen for the study are given in Table I.

Table II. *Expression of tumor markers in relation to serum estradiol and progesterone (n=80).*

	Serum estradiol pmol/L			Serum progesterone nmol/L		
	High staining	Low staining	*p*-value	High staining	Low staining	*p*-value
c-myc ≥50% *vs.* <50%	64.3	87.1	0.50	2.69	1.28	0.01
p53 >0% *vs.* 0	67.2	100.5	0.31	1.40	2.51	0.046
EGFR						
≥20% *vs.* <20%	54.6	194.6	0.0007	1.87	1.18	0.33
CD4+ ≥20% *vs.* <20%	83.8	47.1	0.35	1.60	1.91	0.66
VEGF						
≥50 *vs.* <50%	85.9	61.3	0.49	2.06	1.34	0.23
E-cadherin intensity moderate/high						
vs. none-low staining	69.6	109.2	0.27	1.81	1.96	0.81
CD44 ≥50% *vs.* <50%	83.7	69.6	0.69	1.69	2.22	0.38
Ki-67(MIB-1) ≥50% *vs.* <50%	90.1	71.8	0.57	1.83	1.87	0.95
p27 >0% *vs.* 0	75.4	96.1	0.61	1.49	2.30	0.11
Cyclooxygenase-2 (COX-2) intensity low-high *vs*. absent	88.7	48.5	0.31	1.93	1.52	0.54

EGFR: epithelial growth factor receptor; VEGF: vascular endothelial growth factor.

The biopsies were evaluated by an external senior pathologist, who was blinded for clinical details. A four-grade semi-quantitative score was used, where 0 was absence of biomarker expression, 1 was expression in 1-19% of cancer cells, 2 was 20-49% and 3 was 50% or more cells with expression of the tumor marker. For E-cadherin and COX-2, intensity of staining (absent, mild, moderate and severe) was more useful. CD4+ was evaluated by the degree of expression that surrounded the cancer cells. Aberrant staining of the cytoplasm was registered, but was in general closely related to nuclear or membranous staining. The results were only insignificantly altered when aberrant staining was considered. Due to technical reasons there were occasional cases (one to four) where an individual biomarker could not be diagnosed in individual patients.

The best explanatory cut-off level was used when the results of biomarker staining were dichotomized. When there was no evidence of any association with any variable, dichotomization was made so that a similar number of patients were included in the two groups. A Chi^2 test (likelihood ratio) was used to estimate *p*-values for categorical variables and the *t*-test was used for continuous variables. With the exception of VEGF, there were no tendencies for different antibody staining with different clinical stages. VEGF was expressed in 78% of stages IB/IIA and in 60% of IIB/IV, respectively ($p=0.03$).

The study was approved by the Research Ethical Committee, Medical Faculty, Umeå University, Sweden.

Results

The mean age was 59.7 years with 40 pre- and 88 postmenopausal women. The majority (87.5%) of the women had experienced childbirth (mean parity 2.7) and 46% were current smokers, when ex-smokers were excluded. Clinical staging revealed 52 (40.6%) cases of stage IB, 15 (11.7%) stage IIA, 19 (14.8%) stage IIB, and 42 (32.8%) cases of stages III-IV. The tumor grades according to WHO classification were highly (14%), moderately (60%) and poorly (22%) differentiated.

Associations between serum estradiol and progesterone, and the tumor markers are given in Table II. Low expression of EGFR was significantly associated with high serum estradiol. High progesterone levels were associated with a high c-myc expression, but not with p53 expression. In fertile women, serum hormones with high and low c-myc expression were sampled on menstrual cycle day 11.6 and 12.8, respectively ($p=0.78$). The corresponding values for p53 were on day 11.4 and 14.0 ($p=0.35$). The associations with hormones were relatively higher in fertile women. Thus, serum progesterone was increased more than two-fold (4.63 nmol/L *vs.* 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 *vs.* 2.08 nmol/L; $p=0.10$), as compared to the remaining study population. The lack of significant *p*-values might be caused by the low number of available fertile women (n=23). Expression of the tumor markers was not associated with age.

Current smoking was strongly associated with the absence of p53 expression (Table III). History of oral contraceptive use was not associated with any marker.

Discussion

The major findings of this study were the significant association between an absence of p53 and a high expression of c-myc, and increased serum progesterone levels; high expression of EGFR and low serum estradiol; and absence of p53 and smoking. Although never investigated previously in relation to cervical cancer, these correlations are biologically plausible.

The following seven markers did not show any association with smoking or hormones, *i.e.* Ki-67(MIB-1), p27, CD44, CD4+, E-cadherin, VEGF and COX-2.

Table III. *Expression of tumor markers in relation to smoking (n=102) and duration of oral contraceptive use (n=120).*

	Current smoker (n=46) No. (%)	[1]Current non-smoker (n=56) No. (%)	*p*-value	OC use <12 months (n=86) No. (%)	OC use ≥12 months (n=34) No. (%)	*p*-value
p53 >0%	20 (33.9)	26 (60.5)	0.008	53 (61.6)	18 (52.9)	0.38
Cyclooxygenase-2 (COX-2) intensity low-high	31 (40.3)	15 (60.0)	0.09	59 (70.2)	28 (82.4)	0.16
VEGF ≥50%	29 (61.7)	41 (77.4)	0.09	55 (65.5)	28 (82.4)	0.06
CD4 ≥20%	33 (70.2)	40 (76.9)	0.45	26 (30.6)	9 (31.0)	0.96
E-cadherin intensity moderate-high staining	30 (41.7)	15 (55.6)	0.22	55 (66.3)	24 (72.7)	0.50
CD44 ≥50%	33 (70.2)	40 (71.4)	0.89	56 (65.1)	25 (73.5)	0.37
Ki-67 (MIB-1) ≥50%	18 (40.0)	25 (45.5)	0.58	35 (41.7)	18 (54.6)	0.21
p27 >0%	34 (77.3)	47 (83.9)	0.40	71 (84.5)	27(84.4)	0.98
EGFR ≥20%	39 (81.3)	42 (80.8)	0.95	69 (85.2)	24 (75.0)	0.21
c-myc ≥50%	26 (56.5)	33 (60.0)	0.72	29 (34.5)	16 (47.1)	0.21

[1]Ex-smokers excluded. EGFR: epithelial growth factor receptor; VEGF: vascular endothelial growth factor.

The proteins studied influence a variety of functions in carcinogenesis. Ki-67 (MIB-1) has been widely used in clinical cancer research for almost 20 years as a proliferation marker (17). In addition to p53, another protein involved in cell cycle arrest was investigated, *i.e.* p27 (18). CD44 is a heterogeneous, polymorphic family of cell-surface glycoproteins that are involved in cell adhesion (19). CD4+ T-helper cells play a crucial role in cell-mediated immune response, *e.g.* towards HPV-infected cervical cells (20), and in response to HPV vaccines (21). Among several proteins responsible for intercellular adhesion, one of the most studied is E-cadherin. Loss of E-cadherin expression has been correlated with loss of differentiation, invasiveness, metastatic potential and poor prognosis (22). VEGF is the major protein that induces angiogenesis. Tumor vasculature, however, is structurally and functionally abnormal and might be dysfunctional with regard to oxygenation of the tumor (23). COX-1 and COX-2 are key regulatory enzymes that catalyse the first step in the synthesis of prostaglandins from arachidonic acid. COX-2 responds to a variety of mitogenic and inflammatory stimuli and is involved in a number of steps in cancer development by itself or *via* prostaglandins (24).

In this study, absence of p53 protein expression was associated with increased serum progesterone and smoking. In tumors where there was no p53 expression, serum samples were collected 3.4 days later in the menstrual cycle. Although not significant, this could be associated with slightly higher serum progesterone levels. It is, however, unlikely that p53 should influence ovarian production of progesterone, which indicates that the reverse is a better explanation. It is also unlikely that the menstrual cycle as such, independent of hormones, should influence p53 expression in cervical cancer. p53 is activated in response to DNA damage and causes cell cycle arrest by blocking the cell at the G1/G2-phase prior to DNA replication and mitosis, and thereby aids in the repair process and prevents mutations. It is a major tumor suppressor protein but is also involved in promoting apoptosis (25). p53 is generally compromised during tumorigenesis. In cervical cancer, the human papillomavirus oncogene E6 is able to promote p53 degradation (26). It has been proposed that sex steroid hormones, in particular progestins, might play a role in this process and the present results lend support to this theory (27, 28).

Cancer types differ by etiology, predisposing factors, histology and aggressiveness. Therefore, comparisons between cancer types are of limited use. However, the finding in this study that absence of p53 expression was associated with smoking is similar to a previous study that showed that exposure to smoke increased expression of dysfunctional p53 in gastric cancer in ferrets (29). Similarly, a study on breast cancer found that progesterone down-regulated p53 expression (30).

The association of an absence of p53 expression with smoking and serum progesterone was supported by our previous findings of an increased S-phase fraction (mitosis) in smokers, as compared to non-smokers, and in fertile women with serum progesterone ≥2.6 mol/L as compared to those with lower levels (4), and a decreased duration of survival of premenopausal women with a low serum estradiol/progesterone ratio who eventually died from cervical cancer (5). Both the present and previous findings support a hypothesized etiological role of sex steroid hormones and smoking in cervical cancer.

We also found a significant association between high expression of c-myc and increased serum progesterone levels. c-myc is one of the 'classic' oncogenes. Its functions are still not completely understood as c-myc binds to hundreds of potential target genes. It is evident that c-myc expression contributes to increased proliferation, loss of

differentiation and, unexpectedly, apoptosis (31). In breast cancer it has been reported that progestins stimulate early cancer with concomitant increased c-myc expression in cell cultures and a progestin regulatory region has been found in the c-myc gene (32). This lends support to the present findings.

EGFR is an established marker for tumor proliferation. It regulates differentiation and has so far been considered a target for therapeutic agents with modest results (33). In this study, low serum estradiol was associated with high EGFR expression, which is not in line with other hormone-dependent cancers. In breast cancer, both estrogen and epidermal growth factor are potent mitogens. There is evidence that they utilize divergent signalling mechanisms (34). The ability of estrogens to induce proliferation of the uterine endometrium is well-known (35). There are some *in vitro* studies in cervical cancer cell lines, where estrogens have been added in different modes and proliferation of the tumor was evaluated. There have been some indications of increased proliferation in certain circumstances (36, 37). However, these were *in vitro* studies and estrogens might have effects not involving EGFR.

The present study showed some intriguing results which lend molecular support for a biological role of some possible co-factors to HPV in squamous cell cervical cancer. The associations that were found were biologically plausible. High expression of c-myc and an absence of p53 expression in women with high serum progesterone could explain previous findings of an increased S-phase fraction with increased serum progesterone. Low EGFR was associated with high serum estradiol and supports previous indications of a slower tumor growth when serum estradiol is high. Finally, smoking was associated with an absence of p53 expression that would contribute to previous findings of an association between smoking and a high S-phase fraction. Our findings lend support to the idea that high concentrations of nicotine and the detection of tobacco-specific nitrosamines in cervical mucus are of biological importance.

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