Predicting the Outcome of Squamous Cell Carcinoma of the Uterine Cervix Using Combinations of Individual Tumor Marker Expressions

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Abstract. *Aim: To evaluate if combining the individual expression patterns of biomarkers targeting different molecular alterations in tumor development will improve prognosis prediction in invasive squamous cell carcinoma of the uterine cervix. Patients and Methods: Ten-year follow-up results in 128 women with cervical cancer were compared to the expression of 10 relevant tumor markers, assessed with immunohistochemistry. The markers were selected to represent cell proliferation, tumor suppression, cell–cell adhesion, angiogenesis, apoptosis, inflammation and immune response. All analyses were adjusted for stage. Results: p53 expression, and low expression of c-myc and COX-2 correlated significantly with 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 with 10-year survival. The overall 10-year survival rate with a low COX-2 and a high CD4+ expression was 76% versus 53% in the remaining women (odds ratio 3.73, 95% CI 1.42-11.0). The survival rate with absent p53 and high COX-2 expression in the tumors was 42% versus 71% (odds ratio 0.25, 95% CI 0.10-0.37), while the corresponding figures for the combination of high COX-2 intensity and expression of c-myc were 27% versus 62% (odds ratio 0.13, 95% CI 0.02-0.52). None of the single markers correlated significantly with outcome in the final Cox regression analyses, while five combinations did. Conclusion: Combinations of selected, biologically plausible tumor markers might be more useful for predicting the outcome than using single markers.*

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Biological tumor markers have been widely used in cancer research during the last decades. The steadily increasing number of proteins recognized to be up-regulated during carcinogenesis or tumor progression have led to development of a large number of immunohistochemical markers representing promising adjuncts in diagnosis and differential diagnosis of cervical neoplasia (1). A diagnostically useful biomarker does, however, not necessarily give prognostic information. Expression of a large number of individual biomarkers has been proposed to have prognostic value often with enthusiastic initial reports, but subsequent studies have frequently shown disappointing or contradictory results (1).

Carcinogenesis is a complex and stepwise process, which involves malignant transformation, insensivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (2). Each of these processes is the result of numerous cellular events that are not yet fully understood. There are three major areas of genetic modification that enable malignant transformation, *i.e.* mutations of protooncogenes, tumor suppressor genes and stability genes (3). Tumorigenesis is not the cause of a single gene alteration but of a number of mutations. Mutations by protooncogenes might lead to cell replication that will not be blocked by tumor suppressor genes. Dysfunctional stability genes, caretakers, will not be able to repair defect DNA.

Most previous immunohistochemical studies on biomarkers have chosen one or more tumor markers and analyzed possible correlations with prognosis. The tumor markers have either been 'new' or chosen to confirm the results of previous studies (3). No systematic approach for the selection of biomarkers that influence different steps of cervical carcinogenesis has been attempted in cervical cancer. The purpose of the present study was to evaluate the

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
p-53	Cell cycle arrest, apoptosis DNA repair	Nucleus	DO-7	1:200	30 min	DakoCytomation	TED pH 9 DAKO
p-27	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 45 minutes in a 96˚C water-bath; EFGR: epithelial growth factor receptor; VEGF: vascular endothelial growth factor; TED: DAKO TED pH 9 S2367; TRS: DAKO TRS pH 6.

clinical outcome, measured as overall 10-year mortality rate, and temporal survival trend by combined biomarker expression in squamous cell carcinoma of the uterine cervix (SCC). Individual biomarkers were chosen to reflect different alterations in cancer development (Table I) and had proven prognostic information in at least one previous report. After a crude analysis of each individual tumor marker, we tested the hypothesis that combinations of the most promising single markers would yield better prognostic information.

Patients and Methods

The study population consisted of 128 women with invasive squamous cell 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 classification (4). All women were followed-up for ten years.

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 blocks corresponding to the marked area and joined into TMA paraffin blocks, containing 25 punch biopsies on average. Each TMA block also included two controls, containing human tissue, as specified by the producer. The microscopic evaluation included the complete TMA-biopsy.

Immunohistochemistry was performed at the Department of Pathology and Clinical Cytology, as described elsewhere (5). In brief, 3 μm-thick sections from the TMA blocks were cut and rehydrated. Immunohistochemical staining was carried out 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 routinely. Details of the ten antibodies chosen for the study are given in Table I.

All ten antibodies were evaluated by an external senior pathologist (AL; see acknowledgements), 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, however, intensity of staining (absent, mild, moderate or intense staining) was used, as 93% of the cells showed some E-cadherin expression. This was also true for COX-2. Evaluating intensity was based on the pathologist's long experience of immunohistochemistry. CD4+ was evaluated in the area surrounding the cancer cells. Due to technical reasons there were occasional cases (one to four per biomarker) where an individual biomarker could not be evaluated in individual patients. Staining was evaluated in the nucleus, cytoplasm, or cell membrane, as appropriate (Table I, Localization).

There is no general consensus for the cut-off levels of the tumor markers that were investigated in the study and the best cut-off level for discrimination between a favorable or poor prognosis was therefore used when the results were dichotomized. When there was no evidence of any prognostic importance (Table II) (EGFR, CD44, Ki-67 (MIB1), VEGF, E-cadherin and p27), dichotomization was made at the median number of patients.

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

	Survival, cases[1] No. (%)	Survival, comparisons[1] No. (%)	Odds ratio[2]	95% CI[2]	*p*-value[2]
p53 >0% (n=77) *vs.* 0% (n=50)	50 (65.8)	25 (49.0)	2.88	1.27-6.87	0.01
CD4 ≥20% (n=37) *vs.* <20% (n=86)	26 (70.3)	46 (53.5)	2.28	0.94-5.86	0.08
E-cadherin intensity moderate/high (n=87) *vs.* none-low (n=37)	55 (63.2)	19 (51.4)	1.32	0.55-3.13	0.52
VEGF ≥50% (n=87) *vs.* <50% (n=38)	55 (63.2)	19 (50.0)	1.16	0.48-2.73	0.73
EGFR ≥50% (n=101) *vs.* <50% (n=22)	59 (58.4)	14 (63.6)	1.13	0.39-3.20	0.81
CD44 ≥50% (n=88) *vs.* <50% (n=40)	51 (58.0)	24 (50.0)	1.05	0.45-2.43	0.91
Ki-67 ≥50% (n=68) *vs.* <50% (n=56)	32 (57.1)	41 (60.3)	0.93	0.42-2.09	0.87
P27 >0% (n=103)vs. 0% (n=21)	60 (58.3)	13 (61.9)	0.52	0.17-1.50	0.23
c-myc ≥50% (n=47) *vs.* <50% (n=79)	23 (48.9)	51 (64.6)	0.41	0.18-0.93	0.04
Cyclooxygenase-2 intensity high (n=23) *vs.* absent/low/moderate (n=103)	10 (43.5)	64 (62.1)	0.29	0.10-0.81	0.02

[1]As compared to the remaining study population; [2]Adjustments were made for stage classified into IB/IIA-IIB/III-IV. VEGF: vascular endothelial growth factor; EFGR: epithelial growth factor receptor;

Overall 10-year survival was analyzed with logistic regression and was used for odds ratios, 95% confidence intervals (CI) and *p*-values adjusted for clinical stage, divided into stages IB/IIA-IIB/III-IV. Combinations with two variables were made for three markers that were significantly correlated with, and for one marker that closely correlated with survival. Cox regression (proportional hazard) was used to analyze temporal trends in survival and these results are given as risk ratio, 95% confidence limits (CL) and *p*-values.

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

Results

Only squamous epithelial carcinomas were included. All women were treated with radiotherapy, forty-four also had surgery and treatment in accordance with contemporary routines. Mean age was 59.7 years and 112 (87.5%) of the women had experienced childbirth with a mean parity of 2.7. Fifty-four (42%) of the tumors were clinically staged IB, 14 (11%) IIA, 18 (14%) IIB, 35 (27%) III and 7 (5%) as stage IV. The mean 10-year survival was 61%. Survival decreased continuously from 79% in stage IB down to 14% in stage IV. Aneuploidy was diagnosed in 51 (52.0%) tumors. Eighteen (14%) tumors were highly, 73 (58%) were moderately, and 30 (24%) were poorly differentiated. Seven tumors could not be classified. Only clinical stage and age correlated with 10-year survival, but age lost its significance when adjustment for stage was made

Individual tumor markers. With the exception of VEGF, there was no significantly different antibody staining between stages. VEGF was expressed in 78% and 60% in stage IB-IIA and IIB-IV, respectively ($p=0.03$). More advanced tumors were seen in elderly women, but as age did not influence the survival within each stage, no adjustments were made. The 10-year survival rate for each biomarker, dichotomised by frequencies of cells stained or, for E-cadherin and COX-2, by staining intensity is given in Table II. Three individual biomarkers were associated significantly with 10-year survival, *i.e.* expression of p53, and low expression of c-myc or COX-2. CD4 expression was associated with increased survival, however non-significantly ($p=0.09$), and was included in the analyses of combinations.

Survival. Ten combinations correlated significantly with overall 10-year survival after adjustment for stage (Table III). 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 (69%), respectively. A strong correlation with overall survival was also evident with the combination of any p53 expression and low c-myc expression. The differences in survival rates between patients having tumors with one of these combinations and the remaining study population varied between 19% and 32%.

Five combinations of biomarkers correlated inversely with survival rate. Of those, four combinations included the 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 with a very poor survival, but this group of women was small.

Table III. *Combinations of tumor markers related to a favourable prognosis in 128 women with invasive squamous cell cervical cancer.*

Combinations		10-year survival				
	No. of cases	Cases (%)	Comparison group (%)[1]	OR[2]	95% CI[2]	*p*-value[2]
COX-2[3] and CD4+ ≥20%	33	25 (75.8)	49 (53.3)	3.73	1.42-11.0	0.01
p53 >0% and/or CD4+ ≥20%	93	60 (64.5)	16 (45.7)	3.56	1.43-9.44	0.008
p53 >0% and/or c-myc <50%	111	69 (62.2)	7 (41.2)	3.36	1.09-10.93	0.04
COX-2[3] and p53 >0%	59	41 (69.5)	35 (50.7)	3.09	1.36-7.45	0.009
c-myc <50% and/or CD4+ ≥20%	88	57 (64.8)	18 (46.2)	2.76	1.18-.67	0.02
p53 =0%and/or c-myc ≥50%	81	42 (51.8)	32 (71.1)	0.32	0.12-0.75	0.02
COX-2[4] and/or p53 =0%	67	32 (47.8)	42 (71.2)	0.25	0.,10-0.37	0.0002
p53 =0% and CD4+ <20%	33	14 (42.4)	60 (64.5)	0.29	0.11-0.73	0.01
p53 =0% and c-myc ≥50%	16	6 (37.5)	68 (62.4)	0.25	0.07-0.79	0.02
COX-2[4] and c-myc ≥50%	11	3 (27.3)	71 (62.3)	0.13	0.02-0.52	0.007

[1]As compared to the remaining study population; [2]adjustments were made for stage classified into IB/IIA-IIB/III-IV; [3]absent, light or moderate staining intensity; [4]high intensity.

The four single markers and the ten combinations were finally analysed for temporal survival trend using Cox regression (Table IV). None of the single markers remained significantly correlated with survival. Five combinations correlated significantly with high or poor survival and three combinations showed borderline significance.

Table IV. *Survival analysis by Cox regression in 128 women with invasive squamous cell cervical cancer.*

Combinations	Risk ratio[1]	95% CL[1]	*p*-value[1]
p53>0% (single)	1.16	0.97-1.39	0.11
CD4+≥20% (single)	1.14	0.94-1.39	0.18
p53>0% and/or c-myc <50%	1.27	1.00-1.58	0.05
c-myc <50% and/or CD4+ ≥20%	1.22	1.00-1.48	0.05
COX-2[2] and CD4+ ≥20%	1.21	1.01-1.50	0.05
COX-2[2] and p53>0%	1.21	1.01-1.45	0.04
p53>0% and/or CD4+ ≥20%	1.20	0.98-1.46	0.07
c-myc≥50% (single)	0.86	0.71-1.03	0.10
COX-2[3] (single)	0.81	0.65-1.03	0.09
p53=0% and CD4+ <20%	0.84	0.69-1.04	0.11
COX-23 and/or p53=0%	0.81	0.68-0.97	0.03
c-myc ≥50% and/or p53=0%	0.77	0.61-0.98	0.04
p53=0 and c-myc≥50%	0.77	0.62-0.98	0.04
COX-2[3] and c-myc≥50%	0.70	0.52-0.99	0.04

[1]Proportional hazard. Adjustments were made for stage classified into IB/IIA-IIB/III-IV. Variables in Tables were compared with the remaining study population; [2]absent, light or moderate staining intensity; [3]high staining intensity.

Discussion

The aim of the present study was to evaluate biological markers that have been previously investigated for prognostic information in cervical, as well as other cancers and that covered a variety of major functions in carcinogenesis. The initial step was to evaluate the ten selected biomarkers individually and then to evaluate combinations of markers that correlated with survival. Our hypothesis that the combinations of markers representing different functions in carcinogenesis would give synergistic information about survival was confirmed. Two groups were small (combinations of p53 and c-myc, and COX-2 and c-myc) and their possible clinical value is uncertain. However, they represented the worst outcome in the study.

The four markers that correlated with the outcome represent at least five major steps in carcinogenesis, *i.e.* cell cycle progression (c-myc), tumor suppression (p53), inflammation and apoptosis inhibition (COX-2), and immune response (CD4+), and increased the biological plausibility of the results. Six tumor markers (EGFR, Ki-67 (MIB-1), p27, CD 44, VEGF, E-cadherin) did not add any information to survival in this study and were not used for combinations.

EGFR, a glycoprotein located at the cell surface, is over-expressed in a wide variety of cancers. It also regulates differentiation and has been considered a target for therapeutic agents. Some authors found that EGFR expression was related to poor prognosis, while others did not (6, 7). Another marker of proliferation, Ki-67 (MIB-1) has been widely used in clinical cancer research during the last 20 years (8). Recent studies showed that high MIB-1 expression in lymph node metastasis of SCC positively correlated with longer survival, but carried no prognostic information in the primary tumor (9, 10). Our results are concordant with these findings and might be explained by increased radio-sensitivity in highly proliferating tumors.

Among several proteins responsible for intercellular adhesion, one of the most studied is E-cadherin. Loss of E-cadherin expression has been associated with loss of differentiation, invasiveness, metastatic potential and poor prognosis (11). Few studies investigated the prognostic value of E-cadherin in cervical cancer. CD44 is a heterogenous family of cell-surface glycoproteins that are involved in cell adhesion. There are at least 30 different isoforms with different functions (12). CD-44 has not convincingly been shown to be a prognostic marker. It might have been relevant to include the CD44v6 isoform (12-14).

VEGF was the only protein in this study that correlated with clinical stage. VEGF expression decreased with increasing stage. Loncaster *et al.* (15) observed a decreased survival rate, but no difference in local tumor control, with increased VEGF levels in women treated with radiotherapy. It might seem paradoxical as vascularisation of the tumor is necessary for growth. High levels of VEGF expression do, however, not necessarily mean that the tumor is adequately oxygenated (16).

C-myc is one of the 'classic' oncogenes and its translocation in Burkitt's lymphoma was first shown in 1982 (17). The functions of c-myc products are still not completely understood as they bind to hundreds of potential target genes. It is however evident that c-myc expression contributes to increased proliferation and loss of differentiation (18). The findings of c-myc protein as a prognostic factor have been contradictory. Brenna *et al.* (19) did not find any prognostic value independent of FIGO stage, while Soh *et al.* did (20). In the present study, c-myc as an individual marker and in particular with combinations proved valuable.

p53 was initially known to induce cell-cycle arrest at the G1 and G2 checkpoints prior to DNA replication and repair of damaged DNA, but also for inducing apoptosis, thereby hampering development of cancer cells. Over the years, the 'p53 story' has become increasingly complex (21). It is now known that p53 activation after several signals of cellular stress or DNA damage needs to be compromised during tumorigenesis. In cervical cancer, the human papillomavirus oncogene E6 is able to promote p53 degradation (22). In contrast to the present study, other studies did not find any significant correlation between p53 expression and prognosis (9, 10, 23). These three studies focused on early stage cancer, which at least partly might explain the diverging results. p27 is another cyclin-dependent kinase inhibitor and eventually causes cell cycle arrest, most importantly in the G0/G1-phase, but has usually not been related to prognosis in cervical cancer although there are contradictory results (24-26).

Cyclooxygenase-2 (COX-2) was included in the study because of its correlation to increased inflammatory response in tumors (27). In addition to the present study, increased COX-2 expression has been associated with poor prognosis in two recent studies on SCC (6, 25). Gaffney *et al.* (6) evaluated intensity and frequency to produce a COX-2 score. COX-2 was significantly correlated with disease-free, but not overall survival. In accordance with the present study, Ferrandina *et al.* (28) reported a poor prognosis with increasing COX-2 staining of tumor cells, but also a favorable prognosis when was COX-2 present in the stroma. The authors also found lower CD4+ expression concomitantly with high tumor-cell COX-2 expression, suggesting participation of COX-2 in inhibition of immune response (28). Similar results for CD4+ have been reported (29). In the present study there were very good survival estimates when low COX-2 levels and high CD4+ levels were combined. It is now well accepted that CD4+ T-helper cells play a crucial role in cell-mediated immune response, *e.g.* towards HPV-infected cervical cells and in response to HPV vaccines (30-31).

The disturbing discrepancies in results between different studies can reflect technical, interpretative or biological impacts. Cheuk and Chan (32) suggest that reluctance of the interpreters to delineate nuclear, cytoplasmic, membranous and stromal staining may also influence the study results. Aberrant staining might signal dysfunctional proteins. In the present study, subcellular localization of staining was considered, in particular aberrant cytoplasmic expression, but it did not influence the results substantially. For two of the proteins, E-cadherin and COX-2, the intensity rather than the extent of staining was discriminatory.

Considering the complexity of tumor development involving so many steps and functions, any search for a single marker that adds prognostic information seems unrealistic. Our initial idea that some combinations of markers would improve the prediction of survival was confirmed in the present study. Demonstrating combinations of tumor markers correlated with survival may also widen our knowledge about the complex interaction of different proteins in carcinogenesis. Such examples are the relation between the expression of an oncogene (c-myc) and a tumor suppressor (p53), or immune response (CD4+), as compared to inflammation with subsequent angiogenesis (COX-2).

The marker combinations carrying prognostic information may also represent a contribution to clinical staging placing the patients into good or poor prognostic categories, but is at present speculative. Some conditions should be fulfilled before evaluating an instrument for prognostic information. Inevitably, combinations that are used must be biologically plausible, which was the case of the four biomarkers included in the final analyses. The difference in survival between cases who test positive and those who test negative must be large enough to be clinically important. Each group must have a substantial relative size. One group in this study only included 9% of the study population while several included more than 50% of the patients. Finally, there should be a clearly significant and absolute difference between the two groups.

Some combinations of biomarkers in this study could be of special interest for clinical outcome, *e.g.* for the

prediction of prognosis. Combination of low COX-2 expression with simultaneous CD4+ expression gave large differences in survival rate between cases and the comparison group indicating that low inflammatory reaction with high immune response gives a favourable prognosis. Low COX-2 expression and manifestation of p53 also gave large differences in survival. The presence of either tumor suppression (p53) or immune response (CD4+) (or both) seemed to be correlated to survival. This combination was present in a majority of the patients.

Decreased survival was seen with a number of biomarker combinations. Loss of both p53 and CD4+ expression gave an absolute 22% difference in mortality rate as compared to the other women. This might be a reflection of the importance of simultaneous tumor growth and poor immunological defence. A similar difference in mortality rate was observed when the oncoprotein c-myc expression was high and tumor suppression (p53) was absent (25% absolute difference in mortality rate). This could represent a very unfavourable situation in carcinogenesis. Large differences in mortality rate (23%) were also found when there was high intensity staining of COX-2 or loss of p53 expression, or both. Similar absolute differences but less significant were found in tumors with loss of CD4+ expression and high c-myc expression. This might be a condition with rapid tumor growth and low immunological response.

Our hypothesis was further supported by the absence of significant correlations between any of the four single markers and survival when analysed using Cox regression. This was in contrast to five combinations that were significantly correlated with outcome, and three combinations of "borderline significance" (95% confidence limits included 1.0 and $p=0.05$). As Cox regression takes survival-length into account and also includes adjustment for clinical stage, it gives important information in addition to the analysis of 10-year overall survival. Substantial correlations are therefore necessary to reach significant differences.

There were four proteins with oncogenic (c-myc, COX-2), suppressive (p53) or immunological (CD4+) properties that correlated to survival in cervical cancer when adjusted for stage. Several combinations of expression of these tumor markers yielded significant differences, both in overall survival rates and with Cox regression analyses, and the results were biologically plausible. The present approach of the evaluation of a combination of tumor markers might be clinically valuable and will be used in further studies.

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