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# Pitfalls in immunohistochemical validation of tumor marker expression — Exemplified in invasive cancer of the uterine cervix

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**Abstract**

*Objectives.* To study if immunohistochemical expression of tumor markers as prognostic predictors is influenced by clinical stage, adjustments for expression of other tumor markers and histological type in cervical cancer.

*Methods.* The study included 129 women with squamous cell cancer and 29 women with adenocarcinomas. Expression of 9 tumor markers relevant for cervical cancer and selected to represent different mechanisms in carcinogenesis was analysed. These were Ki-67, c-myc, LRIG1, p-53, p-27, CD44, VEGF, Cox-2 and CD4+.

*Results.* In late-stage cancer a higher number of tumor-infiltrating CD4 positive cells were associated with a favourable prognosis while a higher Ki-67 index with a poor prognosis. In early-stage cancer a high LRIG1 expression was associated with a favourable prognosis. Significantly different expressions were found at early-stage versus at late-stage squamous cell cancer for VEGF, p27 and LRIG1 which were all more strongly expressed in early stages. Adjustments for all selected tumor markers and clinical stage converted VEGF and LRIG1 expression from non-significant to significant prognostic predictors while the association between p53 expression and good prognosis was strengthened. Adjustments for Cox-2 and c-myc had the strongest impact on VEGF as a prognosis predictor and LRIG1 was most influenced by adjustment for p53. All correlations became non-significant when women with adenocarcinoma and other invasive tumor types were included.

*Conclusions.* Failure to analyse clinical stages separately, failure to adjust for expression of relevant concurrent tumor markers and inclusion of different histological subtypes into the same study group may lead to false conclusions regarding the significance of prognostic tumor markers.
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*Keywords:* Uterine cervical cancer; Tumor markers; Interactions; Clinical stage; Histological type

## Introduction

Immunohistochemical detection of tumor markers has become a widely used method in research and routine. Numerous studies have been carried out to evaluate the diagnostic, prognostic and therapeutic significance of these cellular proteins. While increasingly more tumor markers are commercially available and evaluated, often with conflicting results reported, consensus for the clinical use of tumor markers in different cancer types is generally lacking (with the exception of a very limited number of antibodies). Methods exploring the genetic signatures of cancer types for prognosis prediction, such as that generated for breast cancer [1] are now expected to replace immunohistochemical tumor marker detection. However, these methods are not yet established. When different genetic studies are compared, the gene sets are largely non-overlapping and these methods will not answer which are the specific biological mechanisms leading to an aggressive cancer [2]. In comparison with the easy immunohistochemical methods gene techniques are not yet available in most clinical laboratories.

In breast cancer tumor markers are often used in clinical practice. Evaluation of estrogen and progesterone receptors to

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predict response to anti-hormonal treatment with tamoxifen has been standard since many years [3]. HER-2/ErbB-2/neu expression is used for predicting prognosis and clinically for the decision making about trastuzumab (Herceptin) treatment, an antibody specific for erbB-2 [4]. E-cadherin expression has been widely used clinically for differential diagnosis between ductal and lobular carcinoma [5]. In cervical cancer there is no similar well-accepted clinical use of tumor marker expression although numerous studies have addressed the issue.

The major difficulty to evaluate studies on tumor markers and their impact in different cancer types is the inability to compare the results of different studies [6]. Some factors are inevitable, such as clinical characteristics, commercial antibodies, laboratories and evaluation of expression. There are, however, major flaws that must be considered when results from investigations should be interpreted. The aim of the present study is to demonstrate some of these flaws, using a material of invasive squamous cell cervical cancer.

## Material and methods

The study population consisted of 165 women with invasive carcinoma of the uterine cervix stage IB to IV, all treated by radiotherapy. Squamous cell carcinoma was diagnosed in 129 women, 29 tumors were adenocarcinomas and 7 women with carcinomas of other histological types were found. The women were admitted to the Department of Gynecologic Oncology, Norrlands University Hospital, Umeå during 1984 to 1990. Clinical staging was made according to FIGO [7] and clinicopathological details were recorded. The women were followed-up for at least 10 years. The material has been presented in more detail elsewhere [8].

Nine tumor markers, representing different major functions in cancer, which could have prognostic relevance in cervical cancer were chosen [9–17]. The tumor markers reflected malignant transformation (c-myc), proliferation (Ki-67), cell cycle arrest (p53, p27), tumor suppression (Leucine-rich Repeats and ImmunoGlobulin-like domains 1 — LRIG1), cell–cell adhesion (CD44), angiogenesis (vascular endothelial growth factor — VEGF), prostaglandin synthesis (cyclooxygenase-2 — COX-2) and immune response (CD4).

Three-micrometer sections of the original paraffin blocks were reviewed by one of the authors (TT) and the most representative area(s) was marked for tissue microarray (TMA). Three-millimeter punch biopsies were taken from the donor blocks and joined into recipient TMA paraffin blocks, containing an average of 25 punch biopsies. Each TMA block also included two controls from human tissues, as specified by the supplier.

Immunohistochemical staining of LRIG1 was carried out at the Department of Oncology, Norrlands University Hospital, Umeå. The remaining tumor markers were stained at the Department of Pathology and Clinical Cytology, Falun Hospital. In brief, three-micrometer-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.

The biopsies were evaluated by an external senior pathologist who was blinded for clinical details. A four-grade semiquantitative score was used, where 0 was the absence of biomarker expression, 1 was the 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 LRIG1 and COX-2, the intensity of staining (absent, mild, moderate and severe) was more useful as the staining was diffuse. Aberrant staining was registered. Due to technical reasons there were occasional cases (one to four) where an individual biomarker could not be diagnosed evaluated in individual patients.

The best explanatory cut-off level for prognosis was used when the results of biomarker staining were dichotomized, as there is no general agreement what cut-off level should be used for these markers in cervical cancer. When there was no evidence of any association to prognosis, dichotomization was made so that a similar number of patients were included in the two groups.

Table 1
Expression of tumor markers, overall 10-year survival, and survival ratios in early-stage and late-stage squamous cell cervical cancer

| | Overall 10-year survival | | Clinical stage IB–IIA, $n=68$ | | | Clinical stage IIB–IV, $n=59$ | | |
|---|---|---|---|---|---|---|---|---|
| | Survival cases no. (%) | Survival comparisons no. (%) | Odds ratio | 95% CI | $p$-value | Odds ratio | 95% CI | $p$-value |
| LRIG1 >0% ($n=61$) vs. 0% ($n=67$) | 42 (68.9) | 33 (49.3) | 4.66 | 1.48–16.7 | 0.008 | 0.88 | 0.30–2.53 | 0.82 |
| CD4 ≥20% ($n=37$) vs. <20% ($n=86$) | 26 (70.3) | 46 (53.5) | 1.12 | 0.35–4.06 | 0.85 | 3.67 | 1.15–12.66 | 0.03 |
| CD44 ≥50% ($n=88$) vs. <50% ($n=40$) | 51 (58.0) | 24 (50.0) | 2.57 | 0.84–7.96 | 0.09 | 0.37 | 0.11–1.17 | 0.09 |
| Ki-67 ≥50% ($n=56$) vs. <50% ($n=68$) | 32 (57.1) | 41 (60.3) | 2.17 | 0.72–7.07 | 0.17 | 0.29 | 0.09–0.90 | 0.03 |
| COX-2 intensity high ($n=23$) vs. absent/low/moderate ($n=103$) | 10 (43.5) | 64 (62.1) | 0.44 | 0.13–1.53 | 0.50 | 0.17 | 0.01–1.04 | 0.11 |
| c-myc ≥50% ($n=47$) vs. <50% ($n=79$) | 23 (48.9) | 51 (64.6) | 0.56 | 0.19–1.68 | 0.30 | 0.33 | 0.09–1.05 | 0.06 |
| p53 >0% ($n=77$) vs. 0% ($n=50$) | 49 (63.6) | 25 (50.0) | 2.56 | 0.86–8.07 | 0.17 | 2.00 | 0.65–6.65 | 0.24 |
| p27 >0% ($n=103$) vs. 0% ($n=21$) | 60 (58.3) | 13 (61.9) | 0.48 | 0.02–3.12 | 0.51 | 0.61 | 0.18–2.06 | 0.42 |
| VEGF ≥50% ($n=87$) vs. <50% ($n=38$) | 55 (63.2) | 19 (50.0) | 1.53 | 0.42–5.22 | 0.50 | 1.23 | 0.42–3.68 | 0.71 |

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