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ESR1 Promoter Methylation in Squamous Cell Cervical Cancer

VERENA KIRN[1], IVANKA ZAHARIEVA[2], SABINE HEUBLEIN[2], FABINSHY THANGARAJAH[1], KLAUS FRIESE[2], DORIS MAYR[3] and UDO JESCHKE[2]

[1]*University of Cologne, Department of Obstetrics and Gynecology, Cologne, Germany;*
[2]*Ludwig-Maximilians-University, Department of Obstetrics and Gynecology, Campus Innenstadt, Munich, Germany;*
[3]*Ludwig-Maximilians-University, Institute of Pathology, Munich, Germany*

Abstract. *Background/Aim: Estrogen receptor-alpha is usually expressed in normal cervical tissue, but its presence is decreased or absent in invasive cervical cancer indicating that its expression is lost during development of invasive cervical cancer. The aim of the present study was to investigate ESR1 promoter methylation in cervical cancer and correlate methylation status with clinico-pathological parameters. Materials and Methods: Fifty patients treated for cervical cancer were included in the study. Isolation and bisulfite treatment of genomic DNA from cervical cancer tissue was performed by commercially-available kits. Methylated ESR1 promoter sequences were detected by quantitative real-time methylation-specific PCR. Results: Methylation status did not present differences regarding age at-diagnosis, FIGO stage, grade, BMI and overall survival for all patients, but within the subgroup of non-keratinizing squamous cell cancer methylation status correlated with grading (p=0.047). Conclusion: Methylation of the ESR1 promoter does not seem to be of any prognostic relevance, but is associated with higher tumor grading of cervical cancer patients.*

Cervical cancer is responsible for 15% of female cancers in developed countries (1). About 80% of these cancers arise from squamous cell dysplasia, whereas 15% are adenocarcinomas and 5% are clear cell adenocarcinomas (2). Knowledge of the molecular mechanisms underlying the development and metastases of cervical cancer, except for human papilloma virus infection, is limited (3). Especially the fact that almost all women undergo HPV-infection at some point during life, but only a small percentage of these develop cervical dysplasia and an even smaller percentage invasive cervical cancer, implies that co-factors exist that influence the development. Known epidemiological co-factors are smoking and multiparity (4).

Promoter-methylation is an epigenetic alteration that can be seen in multiple types of human neoplasias (5). Hyper-methylation of a certain gene usually inhibits gene expression. During the last years methylation of a number of genes has been described for cervical cancer although direct correlations with clinico-pathological parameters are missing (6-8). The fact that methylation rates increase from cervical intra-epithelial neoplasia to invasive cervical cancer supports its role in carcinogenesis (9).

Issa *et al*. (10) first described the methylation of CpG-islands in the promoter of the estrogen receptor alpha (ER-alpha) in colorectal cancer. Non-malignant tissues from thyroid, breast, lung, cervix and prostate were examined for the presence of promoter methylation and found to be negative (11). In contrast, *ESR1* promoter methylation seems to play a role in the early steps of carcinogenesis in several tumor sites including lymphoma, esophageal cancer and colorectal cancer (10, 12, 13), being present in almost 100% of primary colorectal tumors.

ER-alpha is usually expressed in normal cervical tissues, but expression is decreased or absent in invasive cervical cancer indicating that ER-alpha expression is lost during development of invasive cervical cancer (14, 15). Zhai *et al*. demonstrated that restoration of *ESR1* expression in ER-alpha-negative cervical cancer reduced cell invasiveness in cell culture and concluded that loss of ER-alpha expression plays a major role in mediating cervical cancer invasion and progression (16).

The aim of the present study was to investigate *ESR1* promoter methylation in tissue samples of invasive cervical cancer and to correlate methylation status with clinico-pathological parameters such as grade, stage, BMI and survival.

Correspondence to: Verena Kirn, University of Cologne, Department of Obstetrics and Gynecology, Kerpener Str. 34, 50931 Cologne, Germany. Tel: +49 2214784910, Fax: +49 22147886546, e-mail: verena.kirn@uk-koeln.de

Key Words: Cervical cancer, methylation, estrogen receptor, outcome, survival.

Patients and Methods

Study population. A total of 50 patients treated for cervical cancer between 2000 and 2002 were included in the study. Patients were treated at the University Hospital of the Ludwig-Maximilians-University, Department of Obstetrics and Gynecology, according to the actual guidelines based on FIGO stage, including surgery and in some cases adjuvant therapy. Clinical and pathological data were abstracted from patients' charts.

The study was approved by the Ethics Committee of the Ludwig-Maximilians University Munich and was carried-out in compliance with the guidelines of the Helsinki Declaration of 1975. Samples and clinical information were used anonymously.

DNA-Extraction and bisulfite treatment. Serial sections of surgically-resected cervical cancer tissue were analyzed after having been completely processed by formalin fixation, paraffin embedding and routine diagnostics of histopathological examination.

Six serial sections were used for DNA isolation (10 μm) and one section (3 μm) for HE staining. The carcinoma tissue was manually micro-dissected and pooled for DNA isolation to yield one sample per patient. Tissue sections were de-paraffinised in xylene and rehydrated using ethanol in decreasing concentrations. Rehydrated tissue sections were digested using proteinase K dissolved in TE buffer diluted in distilled water. Genomic DNA was isolated using a commercially-available DNA extraction kit (DNeasy; Qiagen, Hilden, Germany). The DNA concentration of each patient sample was measured by OD600 DiluPhotometer (Implen, Munich, Germany) and results are presented in ng/ml. Two micrograms of each DNA sample were treated with sodium bisulphite converting unmethylated cytosine to uracil and leaving methylated cytosine intact, as described previously (17). After bisulphite modification, the DNA was purified and eluted in 20 ml H_2O.

ESR1 promoter analysis. For real-time methylation-specific (rt-MSP) PCR, the following oligonucleotides were used: forward, 5'-ggcgttcgttttgggattg-3'; reverse, 5'-gccgacacgcgaactctaa-3'; TaqMans probe, FAM 5'-cgataaaaccgaacgacccgacga-3' TAMRA. PCR primers and probe were custom synthesized by Applied Biosystems (Foster City, CA, USA). Concentrations were 9 mM for the forward, 3 mM for the reverse primer and 2.5 mM for the probe. Two microlitres of the eluate containing the bisulphite-treated DNA has been used for each rt-MSP. Amplifications were carried out in 96-well plates in a 7000 Sequence detector (Applied Biosystems) in triplets using Universal Mastermix (Applied Biosystems). The reaction volume was 25 μl. Thermal cycling was initiated by denaturation at 95˚C for 10 min. The PCR program followed was 95˚C for 15 s and 60˚C for 1 min for a total of 50 cycles. Each plate contained patient samples and multiple water blanks as well as positive (MDA-MB-435 and MDA-MB-231 cell lines) and negative controls (MCF-7 cell line). The cell lines used were well-characterised regarding the methylation status of their *ESR1* promoter (18). Serial dilutions of the positive control DNA were used to generate a calibration curve for each analysis. To determine the relative levels of methylated *ESR1* promoter DNA in each sample, the values of *ESR1* promoter methylation were compared to the values of the internal reference, the housekeeping gene Cytokeratin-19. Dilution series showed linearity of amplification down to 1:10,000 for rt-MSP for methylated *ESR1* promoter.

Statistical analysis. Statistical analysis was performed by using SPSS 18.0 software (PASW Statistic, SPSS Inc., IBM, Chicago, IL). Correlation analysis of methylation status of the *ESR1* promoter was performed for the histological subtype, tumor stage, grade and BMI with the non-parametric Kruskal-Wallis rank-sum test and the non-parametric Spearman correlation coefficient. Kaplan-Meier curves were drawn for the comparison of survival times. Differences between survival curves were calculated using the chi-square statistic of the log-rank test to test curves for significance. Significance was assumed at $p<0.05$.

Results

From a total of 50 patients 6 had to be excluded from further analysis because tumor tissue was not suitable for DNA extraction and analysis. Mean age at-diagnosis was 46.5 (range=27-69 years) years. Out of the 44 patients 28 had a non-keratinizing squamous cell cancer, 13 had a keratinizing squamous cell cancer and for 3 patients histology was not further specified. Table I presents the patients' characteristics.

Methylation and patient outcome for all patients. Seventeen patients showed positive *ESR1* promoter methylation while 27 did not. Methylation status did not show any difference regarding age at-diagnosis ($p=0.655$), FIGO stage ($p=0.472$), grade ($p=0.241$), BMI ($p=0.522$) and mean overall survival (9.87 years for patients with promoter methylation, 9.66 years for patients without promoter methylation, $p=0.55$).

Methylation and patient outcome within the sub-group of non-keratinizing squamous cell cancer. Within this sub-group of squamous cell cancer 18 patients showed *ESR1* methylation and 10 patients did not. Correlation of methylation status showed the following results: age at-diagnosis ($p=0.666$), FIGO stage ($p=0.259$), grade ($p=0.047$), BMI ($p=0.387$) and mean overall survival ($p=0.960$). Figures 1-3 show boxplot results of Grading, FIGO-stage and BMI.

Methylation and patient outcome within the subgroup of keratinizing squamous cell cancer. Within this sub-group of squamous cell cancer patients, 6 showed *ESR1* methylation while 7 did not. Correlation of methylation status with several factors showed the following results: Age at-diagnosis ($p=0.224$), FIGO stage ($p=0.343$), grade ($p=0.86$), BMI ($p=0.076$) and mean overall survival ($p=0.317$).

Discussion

We established a methylation-specific real-time PCR for the detection of the methylated *ESR1* promoter in squamous cell cervical cancer samples. Methylation status was correlated with FIGO stage, grade, BMI, and survival in order to

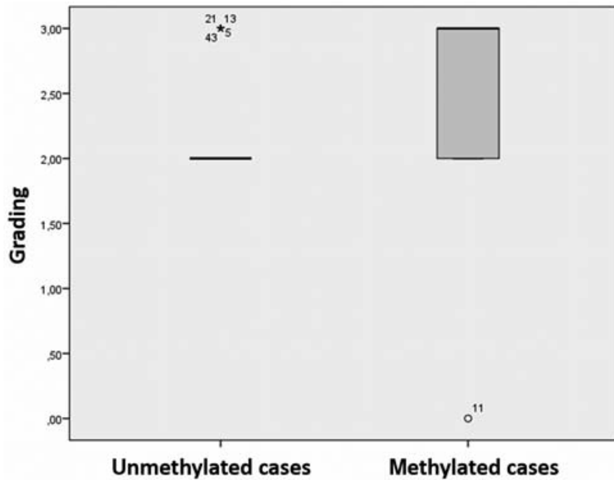

Figure 1. *Grading was significantly different for methylated and unmethylated patients (p=0.047). Boxes represent the range between the 25th and 75th percentiles with a horizontal line at the median. The bars delineate the 5th and 95th percentiles.*

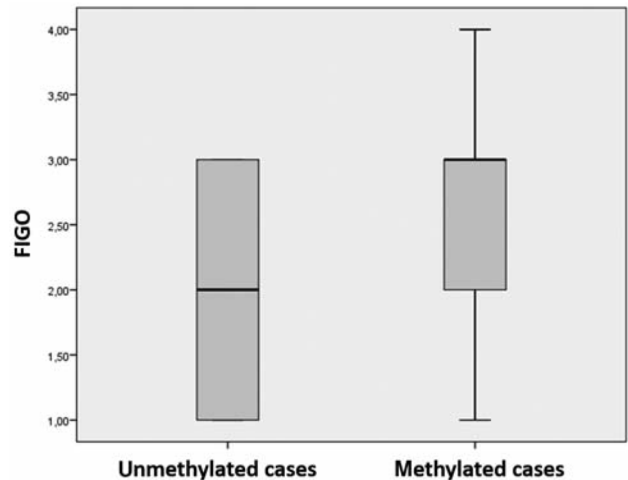

Figure 2. *FIGO-Stage was not associated with methylation status (p=0.259). Boxes represent the range between the 25th and 75th percentiles with a horizontal line at the median. The bars delineate the 5th and 95th percentiles.*

Table I. *Patients' characteristics.*

Patients in final analysis	
(n)	44
Mean age at primary diagnosis	
(years)	46.5
Histology (n) squamous cell carcinoma	
Keratinizing	13
Non-keratinizing	28
Uncertain	3
Tumor grading (n)	
Low grade	1
Intermediate grade	26
High grade	16
Uncertain	1
Tumor stage (FIGO) (n)	
I	15
II	7
III	15
IV	1
Uncertain	6

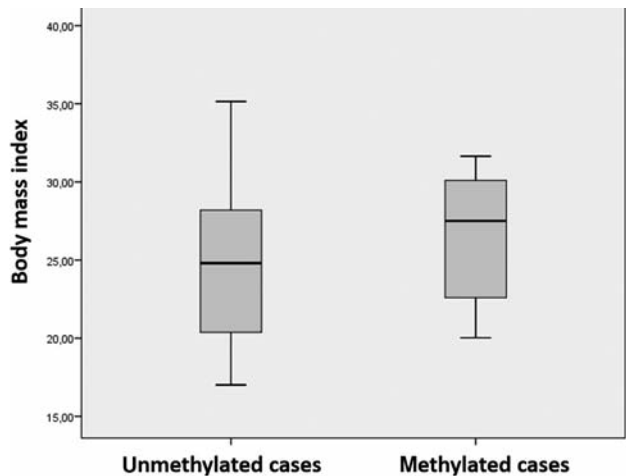

Figure 3. *BMI did not correlate significantly with methylation status (p=0.387). Boxes represent the range between the 25th and 75th percentiles with a horizontal line at the median. The bars delineate the 5th and 95th percentiles.*

investigate its potential as a prognostic marker. Within our study cohort methylation status did not appear to produce any prognostic value. The sub-group of non-keratinizing squamous cell cancer showed significant differences with regard to grading.

Since expression of the estrogen receptor is of importance for the development and invasiveness of cervical cancer, methylation does not seem to be the reason for loss of expression. Zhai *et al*. showed that *ESR1* methylation is in line with loss of expression in cervical cancer cell lines, but not in 10 conventional-PCR-evaluated cervical cancer samples, noting that one has to be careful to transfer results from cancer cell lines to human cancer cases (16).

Several studies investigating gene hypermethylation in cervical cancer (9, 19, 20, 21, 22) have been conducted. These studies include genes such as *DAPK1, FHIT, MGMT, CDKN2A, RAR-beta2, p16, GSTP1, hMLH1, LDOC1, APC, HIC-1,* and *E-cadherin*. Significant higher methylation rates

were found for *DAPK1* and *FHIT* in cervical cancer specimens compared to normal controls, for example, hypermethylation of *LDOC1* correlates with loss of gene expression. Müller *et al*. analyzed the methylation status of 25 genes, including *ESR1*, and found that certain combinations of methylated genes were able to predict for survival (23). Other researchers tried to use promoter methylation as a screening tool for cervical cancer and analyzed promoter methylation in cervical scrapings of patients with cervical cancer and cervical neoplasia (24). In that study *CALCA, DAPK, ESR1, TIMP3, APC* and *RAR-beta2* promoters were significantly more often hypermethylated in cancer cases than in controls. A combination of four genes was able to predict 89% of the cancer cases. *ESR1*-alone was hyper-methylated in 64% of all cancer cases, but only 28 patients were included, therefrom 8 with adenocarcinoma of the cervix, which makes comparison with our results difficult.

Widschwendtner *et al*. examined the methylation status of *CALCA, MYOD1, PGR, TIMP3*, and *hTERT* in serum samples of cervical cancer patients (6). All genes found to be methylated in serum samples were also methylated in the corresponding tissue sample, but only serum DNA methylation status of MYOD1 showed significant differences of survival.

All these studies confirm that a large number of genes is methylated to a higher degree in cervical cancer compared to normal tissue and that methylation can be detected by reliable methylation-specific PCR. However, the role of promoter methylation in the development of cervical cancer and the prognostic and therapeutic relevance remains uncertain since most of the studies mentioned above did not correlate the methylation status with clinicopathological features or survival or were not able to show significant differences.

Although our findings suggest that promoter methylation of *ESR1* is not of true prognostic value for overall survival, we identified a significant correlation of methylation status and high tumor grade. The question remains if treatment with de-methylating agents might be of any therapeutic relevance. In general, treatment of cervical cancer targeting the estrogen receptor is still under review. Some studies support the role of estrogen in the development of cervical cancer, while others do not (25, 26). In a cohort of patients treated with Tamoxifen or Raloxifene, there was no evidence that long-term Raloxifene use reduces, or long-term Tamoxifen use increases, the risk of cervical carcinoma *in situ* (27). On the other hand, treatment of CIN-bearing mice with Fulvestrant during estrogen treatment prevented CIN lesions from progressing to cancer. Further investigation of the role of estrogen and its receptors in human cervical cancers is indicated (28).

In summary, *ESR1* promoter methylation does not seem to be of any prognostic relevance in squamous cell carcinoma of the cervix. It remains unclear if promoter methylation plays different roles in keratinizing *vs*. non-keratinizing squamous cell cancers of the cervix and this notion needs to be further evaluated. Due to the small patient numbers and long survival times in the herein cervical cancer patients studied, future analyses should be conducted employing larger study cohorts longer follow-up periods in order to make evaluation of survival differences possible.

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