

Cytoplasmic Keap1 Expression Is Associated With Poor Prognosis in Endometrial Cancer

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Abstract. *Background/Aim: Oxidative stress is involved in several carcinogenic pathways. Nuclear factor erythroid 2-related factor (Nrf2), Kelch-like ECH-associated protein 1 (Keap1) and Park7 (DJ-1) are the main regulators of antioxidant enzymes eliminating reactive oxidative species (ROS). The roles of these proteins were studied as potential prognostic factors in endometrial cancer. Materials and Methods: Nrf2, Keap1 and DJ-1 expression in endometrial carcinomas was analyzed immunohistochemically. Correlations between staining patterns and clinical prognostic variables were evaluated. Results: Extensive cytoplasmic Keap1 staining correlated to several factors associated with poor prognosis of endometrial cancer including advanced stage, poor histological differentiation, lymphovascular invasion, pelvic lymph node metastasis and deep myometrial invasion. In multivariate analysis, cytoplasmic Keap1 was a stronger predictor of poor progression-free survival than grade. Nuclear Nrf2 staining was seen in all patients with lymph node metastasis while DJ-1 staining was associated with clinically favourable disease types. Conclusion: Cytoplasmic Keap1 expression indicates poor prognosis in endometrial cancer.*

Endometrial cancer is the most common gynecological cancer in developed countries (1). The majority (90%) of women diagnosed with endometrial cancer are older than 50 years. Five-year survival (including all stages) is excellent, but in cases of advanced or high-risk-early-stage disease it is less than 17% (2). Obesity, diabetes mellitus, hypertension,

late menopause, hyperestrogenism, nulliparity, infertility and genetic predisposition have all been identified as risk factors of endometrial cancer (3). Endometrial carcinoma is traditionally divided into two types based on histopathology and pathogenesis. Type I includes histological grade I and II endometrioid carcinomas and mucinous carcinomas whereas type II includes clear cell, serous, grade III endometrioid and mixed carcinomas. Over 80% of endometrial carcinomas are of type I.

Oxidative stress is a dynamic condition where the amount of reactive oxygen species (ROS) and the antioxidant defence mechanisms are imbalanced (4). Oxidative stress has been proven to be involved in carcinogenesis and prognosis in various malignant and premalignant diseases. There are also various defence mechanisms to counteract the unfavourable effects of ROS (5).

Nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) has become a subject of widespread interest in cancer research. The Nrf2-regulated pathway can be considered as one of the most effective ways of a cell to protect itself against harmful ROS (6). When activated, Nrf2 binds to an antioxidant response element (ARE) together with Maf proteins and induces genes for several enzymes, metal-binding proteins, molecular chaperones and drug transporters which are involved in antioxidant defence mechanisms (7, 8). Under physiological conditions almost all Nrf2 is located in the cytoplasm. Kelch-like ECH-associated protein 1 (Keap1) is a protein that binds to Nrf2 and *via* the ubiquitin proteasome pathway represses it. Under oxidative stress Nrf2-Keap1 interaction is disturbed, which leads to up-regulation of Nrf2 and its accumulation in the nucleus (9). This may help cancer cells to survive by protecting them from oxidative damage caused by chemotherapeutic drugs (10).

DJ-1 (PARK7) is a multifunctional proto-oncogenic protein that identifies oxidative damage. It prevents binding of Keap1 to Nrf2 and activates several antioxidant genes

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Key Words: Nrf2, Keap1, DJ-1, oxidative stress, endometrial cancer.

such as Nrf2-regulated antioxidant enzyme NAD(P)H-dependent quinone oxidoreductase 1 (NQO1) (11). Elevated DJ-1 levels have been found in various cancers such as leukemias, astrocytomas and renal carcinomas (12-14). Notable expression of Nrf2 and DJ-1 has also been seen in non-small-cell lung cancer, where it seems to be associated with poor prognosis (15).

This study aimed to evaluate the prognostic value of the levels of Nrf2, Keap1 and DJ-1 in endometrial carcinoma.

Materials and Methods

The study population consisted of 80 patients diagnosed with endometrial carcinoma: 71 endometrioid carcinomas, 7 serous carcinomas and 2 mixed carcinomas. Tissue samples had been collected in 2003-2011 and were stored at the Department of Pathology, Oulu University Hospital. FIGO classification 2009 was used to determine the stage of the disease. Clinical data were gathered from the patient records of Oulu University Hospital (Table I).

Tissue sections (3.5 µm) in paraffin wax were used for immunohistochemical staining. They were deparaffinized in xylene for 3x3 min, rehydrated in a descending series of alcohol solutions and then rinsed. Antigen retrieval was completed in citrate buffer solution (pH 6) in a microwave oven (800 W 2 min, 150 W 2 min) followed by cooling at room temperature for 20 min. Neutralization of endogenous peroxidase activity was carried out in peroxidase arrest solution (Dako, Glostrup, DK).

The antibodies used were rabbit monoclonal anti-Nrf2 (EP1808Y, Abcam, Cambridge, UK) at 1:300 dilution, goat polyclonal anti-Keap1 (Santa Cruz Biotechnology, Santa Cruz, USA) at 1:100 dilution and rabbit polyclonal anti-PARK7/DJ-1 (Abcam, Cambridge, UK) at 1:10,000 dilution. Dako Envision Kits (Dako, Glostrup, DK) were used for the detection of Nrf2 and DJ-1 antibodies, whereas goat-on-rodent HRP-polymer kit (Biocare GHP516L, Biocare Medical, Concord, CA, USA) was used for Keap1.

Quantitation of staining was performed in both cytoplasm and nuclei of the neoplastic tissues. The extent of moderate or strong staining was defined as: 0=0-5%; 1=6-100%.

Statistical analysis. Statistical analyses were carried out with SPSS 21.0 for Windows software. Fisher's two-sided exact test was used to compare the clinical data and immunohistochemical expression. Kaplan-Meier curves with the log-rank test were applied in the progression-free survival analysis. Overall survival analysis was not produced due to the insufficient number of events during follow up. *p*-Values were considered statistically significant when *p*<0.05. Cox regression analysis was applied in multivariate analysis, where the most important traditional prognostic factors, *i.e.* stage (stage I - II or stage III) and grade (low-grade or high-grade) were included in the model.

Results

Moderate to strong cytoplasmic Keap1 expression (>5%) in malignant epithelium was found in 37 samples (46.3%) (Table II, Figure 1). Cytoplasmic Keap1 was associated with more advanced disease (stage II-III), high grade tumors, non-

Table I. Clinicopathological data of 80 subjects.

| | Mean (range) | |
|---------------------------------|------------------|-------|
| Age at diagnosis | 65 (48-86) | |
| BMI (kg/m ²) (n=75) | 28.8 (18.4-42.6) | |
| Carcinoma type | n | % |
| Endometrioid | 71 | 88.8% |
| Serous | 7 | 8.8% |
| Mixed | 2 | 2.5% |
| FIGO stage | | |
| Ia | 30 | 37.5% |
| Ib | 27 | 33.8% |
| II | 9 | 11.3% |
| IIIA | 3 | 3.8% |
| IIIC | 11 | 13.8% |
| Histological grade | | |
| Low-grade | 64 | 80% |
| High-grade | 16 | 20% |
| Myometrial invasion >50% | 44 | 55.0% |
| Lymphovascular invasion | 41 | 51.3% |
| Recurrence | 16 | 20.0% |
| Deaths | 6 | 7.5% |

endometrioid histology, lymphovascular invasion, pelvic lymph-node metastasis and deep myometrial invasion. The presence of cytoplasmic Keap1 expression was a predictor of poor progression-free survival (PFS) (*p*=0.011) (Figure 2). When stage, grade and cytoplasmic Keap1 expression were all included in Cox regression analysis, none of these factors remained significant, probably as a result of the limited number of endpoints. However, when only grade and Keap1 expression were included in the model, cytoplasmic Keap1 was a more significant predictor of poor PFS (relative risk (RR)=3.423; 95%CI=1.030-11.372; *p*=0.045) than grade (RR=1.610; 95%CI=0.522-4.969; *p*=0.408). Nuclear Keap1 expression was mainly seen in neoplastic epithelium and only a few samples had positive staining in areas with epithelial hyperplasia. Nuclear Keap1 expression was seen only in 4 samples (4.0%), while 76 samples (95.0%) were negative (Figure 1). In contrast to cytoplasmic expression, nuclear Keap1 was not associated with any of the other clinicopathological parameters.

Cytoplasmic Nrf2 expression in malignant epithelium was seen in 63 samples (78.8%) (Table II). Moderate or strong nuclear Nrf2 expression was seen in 54 (67.5%) of the samples and, in particular this staining pattern was recorded in all patients with pelvic lymph-node metastasis (9/9) (Figure 1). Expression of Nrf2 was not associated with any other clinical parameter. Nuclear DJ-1 expression (Figure 1) was observed in 39 samples (48.0%) (Table II). It was associated with low-

Table II. Number of cases showing moderate to strong (>5%) cytoplasmic or nuclear immunostaining in neoplastic epithelium. Significant (and nearly significant) *p*-values are presented.

| | Keap1 | | Nrf2 | | DJ-1 | | Total |
|------------------------------|------------------------------|------------------|----------------------|----------------------------|----------------------|----------------------------|-----------|
| | Cytoplasmic n (%) | Nuclear n (%) | Cytoplasmic n (%) | Nuclear n (%) | Cytoplasmic n (%) | Nuclear n (%) | n (%) |
| Histological type | | | | | | | |
| Endometrioid | 28 (39.4) | 2 (2.8) | 54 (77.1) | 46 (65.7) | 56 (83.6) | 36 (53.7) | 71 (88.8) |
| Non-endometrioid | 9 (100) <i>p</i> =0.0005 | 2 (22.2) | 9 (100) | 8 (88.9) | 7 (87.5) | 3 (37.5) | 11 (11.3) |
| Grade | | | | | | | |
| Low-grade | 23 (35.9) | 2 (3.1) | 49 (77.8) | 42 (66.7) | 50 (83.3) | 33 (55.0) | 64 (80.0) |
| High-grade | 14 (87.5) <i>p</i> =0.00023 | 2 (12.5) | 14 (87.5) | 12 (75.0) | 13 (86.7) | 6 (40.0) | 16 (20.0) |
| Stage | | | | | | | |
| Stage I | 17 (29.8) | 2 (3.5) | 45 (80.4) | 37 (66.1) | 43 (81.1) | 30 (56.6) | 57 (71.3) |
| Stage II-III | 20 (87.0) <i>p</i> =0.000036 | 2 (8.7) | 18 (78.3) | 17 (73.9) | 20 (90.9) | 9 (40.9) | 23 (28.7) |
| Myometrial invasion >50% | | | | | | | |
| Yes | 25 (56.8) <i>p</i> =0.044 | 1 (2.3) | 34 (77.3) | 28 (63.6) | 35 (83.3) | 15 (35.7) | 44 (55.0) |
| No | 12 (33.3) | 3 (8.3) | 29 (82.9) | 26 (74.3) | 28 (84.8) | 24 (72.7) <i>p</i> =0.0022 | 36 (45.0) |
| Lymphovascular invasion | | | | | | | |
| Yes | 25 (61.0) <i>p</i> =0.0080 | 2 (4.9) | 34 (85.0) | 24 (61.5) | 33 (82.5) | 16 (40.0) | 41 (51.2) |
| No | 12 (30.8) | 2 (5.1) | 29 (74.4) | 30 (75.0) | 30 (85.7) | 23 (65.7) <i>p</i> =0.026 | 39 (48.8) |
| Pelvic lymph node metastasis | | | | | | | |
| Yes | 8 (88.9) <i>p</i> =0.0093 | 3 (5.7) | 9 (100) | 9 (100) (<i>p</i> =0.051) | 6 (75.0) | 1 (12.5) | 9 (14.5) |
| No | 21 (39.6) | 1 (11.1) | 39 (75.0) | 35 (67.3) | 43 (86.0) | 25 (50.0) | 53 (85.5) |

depth myometrial invasion and absence of lymphovascular invasion. Cytoplasmic DJ-1 positivity was seen in 63 samples (84%) (Figure 1). No statistically significant correlations were found when the expression patterns of Keap1, Nrf 2 and DJ-1 were compared with each other.

Discussion

Patients with early-stage endometrioid-type endometrial carcinomas have a relatively good prognosis, with 10-year overall survival being 80% on average (16). However, there is still a certain subgroup of patients whose disease relapses. This has raised an urgent need to find some additional prognostic factors for clinical decision-making and for treatment tools. One very promising prognostic marker protein for endometrial cancer is the L1-cell adhesion molecule (L1CAM; CD171), the expression of which in early-stage endometrial carcinoma patients has indicated a more aggressive behaviour of the disease (17). Recently, genomic classification of endometrial cancer has also revealed new prognostic factors in endometrial cancer. This study aimed to find additional prognostic markers to further evaluate factors connected to the behavior of endometrial cancer. ARE –regulation was evaluated by assessing the expression patterns of Nrf2, Keap1 and DJ-1. The staining patterns were compared in relation to clinicopathological features such as tumor size, histological grade, pelvic lymph-

node metastasis, myometrial invasion, lymphovascular invasion and relapse-free survival.

Although Nrf2 has been proven to have protective effects against ROS under physiological conditions, it can also provide proliferation advantage to malignant cells during carcinogenesis. The Nrf2-induced ARE-dependent defence mechanism protects cells under oxidative stress. Nrf2 also provides a growth advantage to malignant cells by inducing the expression of genes that are involved in NAD(P)H regeneration and also the genes that regulate cellular glucose influx and purine generation (18). High levels of Nrf2 expression have been found in type II endometrial carcinomas, which are known to develop chemoresistance (10). In addition, nuclear expression of Nrf2 is associated with poor survival in pancreatic adenocarcinoma, non-small-cell lung cancer, melanoma and gliomas (19-22). Activation of the Nrf2-Keap1-ARE pathway is also related to radiation resistance in lung-cancer patients (23). According to earlier studies, Nrf2 is expressed in most tumors in our study. Interestingly all endometrial carcinomas with lymph node metastasis were positive for Nrf2. Even though sensitivity of Nrf2 as a marker for lymph node metastasis is not high, this finding justifies further studies on the role of Nrf2 as a possible specific marker for advanced tumor stage.

Keap1 was associated with all traditional prognostic factors of endometrial cancer and also an unfavorable PFS. According to our results, elevated expression of cytoplasmic

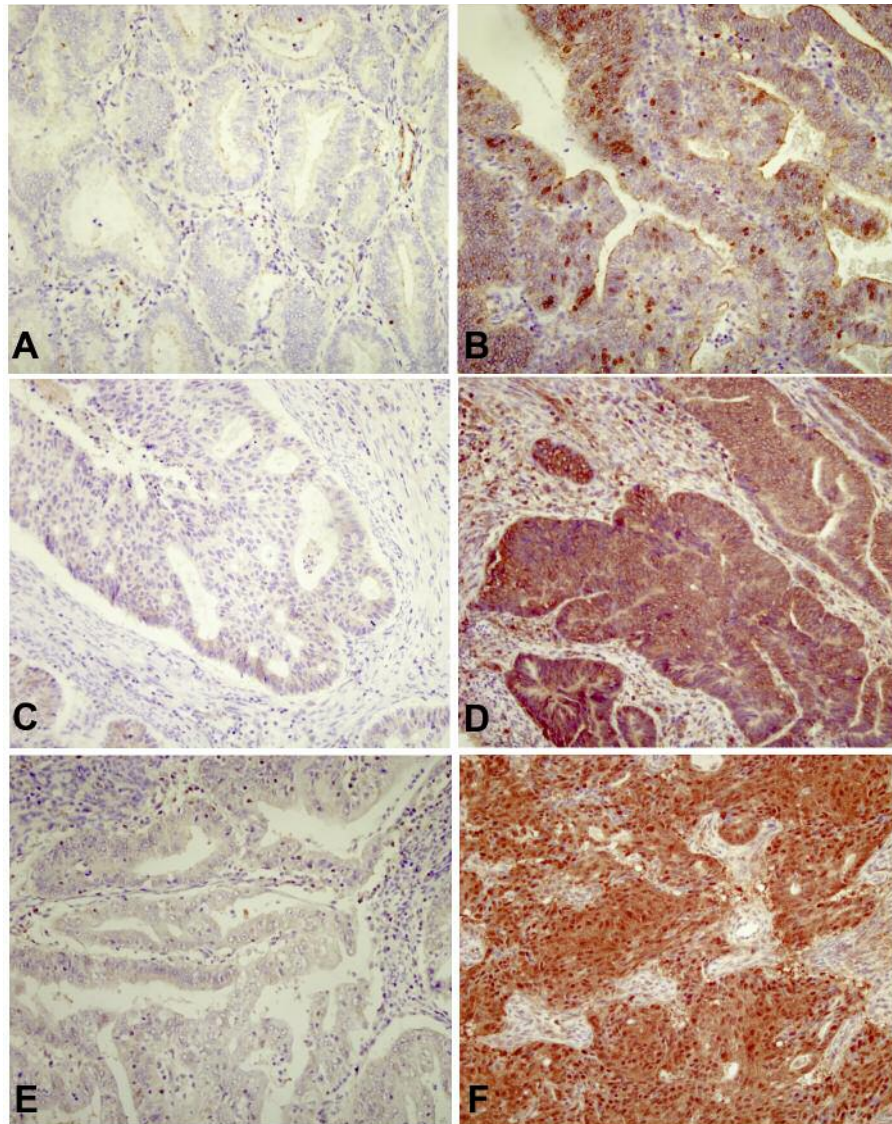


Figure 1. Expression of Keap1 (A-B), Nrf2 (C-D) and DJ-1 (E-F) in endometrial carcinoma. Cytoplasmic Keap1 staining was stronger in high-grade (B) versus low-grade carcinoma (A). Stronger Nrf2 staining was observed in carcinomas with nodal metastasis (D) compared to carcinomas without metastasis (C). Cytoplasmic DJ-1 expression was observed in most carcinomas yet nuclear staining (F) was associated to less advanced tumor stage. Some carcinomas showed no DJ-1 staining (E).

Keap1 is correlated to more advanced disease and invasiveness of the tumor. Correspondingly, elevated Keap1 expression has been associated with a triple-negative phenotype in breast cancer as well as worse survival when patients with estrogen receptor-positive tumours were also taken into account (24). Although Keap1 is considered to be the main regulator of Nrf2, there are also other factors involved in regulating Nrf2 such as, protein kinase C, Jun N-terminal kinase and phosphatidylinositol kinase (25). It is probable that Keap1 overexpression reflects Keap1 induction, particularly in the most oxidatively stressed

tumors. However, our results suggest that Keap1 could be a beneficial prognostic factor in endometrial cancer and in designing adjuvant treatment.

DJ-1 is considered to be a proto-oncogene due to its ability to protect cells against oxidative stress-derived damage by preventing apoptosis (26). Tumor invasiveness and metastatic potential are thought to be associated with epithelial-mesenchymal transition (EMT) generated by suppression of PTEN by DJ-1 (27). Overexpression of DJ-1 has been found in various malignant diseases. It has been linked to invasion and tumor metastasis in nasopharyngeal

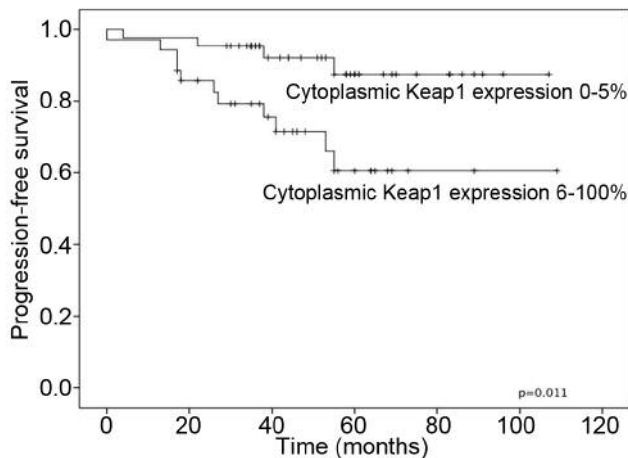


Figure 2. Kaplan–Meier curves comparing low and high cytoplasmic Keap1 expression.

carcinoma and non-small-cell lung-cancer (28, 29). Nuclear expression of DJ-1 has also been found in lung-cancer patients with distant metastases (15). Some recent studies have reported a higher immunoscore in endometrial serous carcinoma *versus* endometrioid carcinoma (30) and increased DJ-1 serum level especially in serous carcinoma (30, 31). In our study, elevated nuclear DJ-1 was associated with the absence of lymphovascular invasion and with lower myometrial invasion. Therefore, it seems that the observed association of DJ-1 with lower stage endometrial cancer gives new perspective to the research field and suggests that the role of DJ-1 as a predictor of unfavourable prognosis is not as clear.

In conclusion, the ARE signalling route in oxidative stress seems to be activated and to be involved in the pathogenesis of endometrial cancer. Clinically the most relevant marker of unfavourable prognosis seems to be cytoplasmic Keap1 expression. Yet, further studies are needed to establish the possible role of Keap1 in clinical decision making. Interestingly, in contradiction to earlier studies with endometrial and other malignancies, DJ-1 was not associated with an aggressive tumor behaviour. Hence, the role of DJ-1 in carcinogenesis is not yet revealed, but needs further study.

Conflicts of Interest

The Authors have stated no conflicts of interest in connection to this article.

Authors' Contributions

Ulla Puistola and Peeter Karihtala contributed to experimental design and conception as well as revision of the manuscript; Juha Kangas and Riikka Salonen contributed to clinical data collection;

Anne Ahtikoski and Juha Kangas contributed to histopathological evaluation and data collection; Peeter Karihtala and Juha Kangas contributed to data analysis; Anne Ahtikoski and Juha Kangas contributed to writing of the manuscript.

Acknowledgements

This study was supported financially by a state subsidy granted to the University Hospital of Oulu. Riitta Vuento and Manu Tuovinen are acknowledged for their contribution to the laboratory work.

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Received January 8, 2019

Revised January 16, 2019

Accepted January 18, 2019