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Asparaginase-like protein 1 is an independent prognostic marker in primary endometrial cancer, and is frequently lost in metastatic lesions

Tina Fonnes [a,b], Hege F. Berg [a,b], Therese Bredholt [a,b], Per-Henrik D. Edqvist [c,d], Kristina Sortland [e], Anna Berg [a,b], Helga B. Salvesen [a,b], Lars A. Akslen [f,g], Henrica M.J. Werner [a,b], Jone Trovik [a,b], Ingvild L. Tangen [a,b], Camilla Krakstad [a,b,*]

[a] Centre for Cancer Biomarkers, Department of Clinical Science, University of Bergen, Bergen, Norway
[b] Department of Obstetrics and Gynecology, Haukeland University Hospital, Bergen, Norway
[c] Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden
[d] Science for Life Laboratory, Uppsala, Sweden
[e] Department of Biomedicine, University of Bergen, Bergen, Norway
[f] Department of Pathology, Haukeland University Hospital, Bergen, Norway
[g] Centre for Cancer Biomarkers, Department of Clinical Medicine, Section for Pathology, University of Bergen, Bergen, Norway

HIGHLIGHTS

- ASRGL1 validates as a prognostic marker in a prospective setting.
- Low ASRGL1 protein and *ASRGL1* mRNA expression predicts poor outcome.
- ASRGL1 expression has independent impact on survival.
- Precursor lesions express high ASRGL1 levels.
- The majority of metastases have low ASRGL1 expression.

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ABSTRACT

Objective. Loss of Asparaginase-like protein 1 (ASRGL1) has been suggested as a prognostic biomarker in endometrial carcinoma. Our objective was to validate this in a prospectively collected, independent patient cohort, and evaluate ASRGL1 expression in endometrial carcinoma precursor lesion and metastases.

Methods. 782 primary endometrial carcinomas, 90 precursor lesions (complex atypical hyperplasia), and 179 metastases (from 87 patients) were evaluated for ASRGL1 expression by immunohistochemistry in relation to clinical and histopathological data. *ASRGL1* mRNA level was investigated in 237 primary tumors and related to survival and ASRGL1 protein expression.

Results. Low expression of ASRGL1 protein and *ASRGL1* mRNA predicted poor disease specific survival ($P < 0.001$). In multivariate survival analyses ASRGL1 had independent prognostic value both in the whole patient cohort (Hazard ratio (HR): 1.53, 95% confidence interval (CI): 1.04–2.26, $P = 0.031$) and within the endometrioid subgroup (HR: 2.64, CI: 1.47–4.74, $P = 0.001$). Low ASRGL1 expression was less frequent in patients with low grade endometrioid primary tumors compared to high grade endometrioid and non-endometrioid primary tumors, and ASRGL1 was lost in the majority of metastatic lesions.

Conclusions. In a prospective setting ASRGL1 validates as a strong prognostic biomarker in endometrial carcinoma. Loss of ASRGL1 is associated with aggressive disease and poor survival, and is demonstrated for the first time to have independent prognostic value in the entire endometrial carcinoma patient population.

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1. Introduction

Endometrial carcinoma is a malignancy originating in the female reproductive tract, and is the fourth most diagnosed cancer in European women after breast, colorectal and lung cancer [1]. Primary surgical

* Corresponding author at: Department of Clinical Science, University of Bergen, Jonas Lies Vei 72, 5020 Bergen, Norway.
E-mail address: camilla.krakstad@med.uib.no (C. Krakstad).

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treatment of endometrial carcinoma is curative for most patients, but 15–20% suffers a relapse within few years [2,3]. Prognosis for recurrent endometrial carcinoma is often poor, especially in patients with systemic recurrence [3]. Little improvement in treatment and survival has been achieved over the last decades, highlighting the need of targeted therapies and more individualized cancer treatment [4]. Discovering, validating and implementing new biomarkers that can identify high-risk endometrial carcinoma patients is crucial as such markers can potentially guide physicians planning treatment for individual patients [2]. Asparaginase-like protein 1 (ASRGL1) is an enzyme classified as a N-terminal nucleophile (Ntn) hydrolase, exhibiting both L-asparaginase and β-aspartyl peptidase activity [5]. It was first described as a novel protein sharing 77% of its genetic sequence with a sperm autoantigen found in rats [6]. The role of ASRGL1 in cancer development and progression is not clear. For several malignancies including mammary, ovarian, and prostate cancer high levels of ASRGL1 in tumor have been reported [7,8]. In endometrial carcinoma loss of the gene encoding ASRGL1 has previously been reported as part of a 29-gene signature associated with features of aggressive disease and poor recurrence-free survival [9,10]. Loss of ASRGL1 in primary endometrial carcinoma has also been suggested to be an independent biomarker for disease-specific survival in a subgroup of patients with endometrioid endometrial carcinoma [11]. In the present study we aimed to validate ASRGL1 as a prognostic biomarker in endometrial carcinoma, and to evaluate the expression of ASRGL1 in clinical specimens from a large, prospectively collected patient cohort including precursor lesions (complex atypical hyperplasias – CAH), primary tumors, and metastases.

2. Material and methods

2.1. Patient series

Women diagnosed with endometrial carcinoma at Haukeland University Hospital, Norway, were prospectively included during the period 2001–2015. Haukeland University Hospital is a referral hospital for Hordaland County, and the patient series is representative of the Norwegian population due to similar incidence rates and patient characteristics for this region compared to the whole of Norway [12]. Primary tumor tissue from 782 patients was collected during hysterectomy and prepared as formalin fixed and paraffin embedded (FFPE) tissue. This corresponds to 76% of endometrial carcinoma patients included in the local biobank during this time period. Cases not represented in TMAs include inoperable patients, patients with sparse tumor material in hysterectomy specimen, and cases with poor technical quality of TMA tissue cylinders. Fresh frozen tissue was collected in parallel when possible. Clinical information was retrieved from medical records as previously described [13], including age at primary treatment, International Federation of Gynaecology and Obstetrics (FIGO) stage (according to 2009 criteria), histopathological type and grade, and follow-up data. Biopsies from precursor lesions were obtained from 90 patients diagnosed with CAH. Samples from metastatic tissue were available for 87 patients (179 lesions in total). All parts of the study have been approved according to Norwegian legislation, including the Norwegian Data Inspectorate, Norwegian Social Sciences Data Services and Western Regional Committee for Medical and Health Research Ethics (REK 2009/2315). All participants were informed and gave written consent prior to inclusion.

2.2. Immunohistochemistry (IHC)

FFPE tissue was used to generate tissue microarrays (TMA) as previously described [14]. The tumor area with highest tumor content was identified on hematoxylin and eosin stained slides. Using a custom made precision instrument (Beecher instruments, Silver Spring, MD, USA) tissue cylinders (0.6 mm) (three tissue cylinders for primary tumors and CAHs and one tissue cylinder for metastatic lesions) were punched out of the donor block and mounted in a recipient paraffin block. TMAs were stained by automated IHC using a previously published polyclonal anti-ASRGL1antibody (HPA029725, diluted 1:375) [11], or a monoclonal anti-ASRGL1 antibody (AMAb90907, diluted 1:1000) (Both; Atlas Antibodies, Stockholm, Sweden). ASRGL1 staining in tumor cells was evaluated without considering sub-cellular localization [11]. A staining index (SI) was calculated for each patient based on all three cylinders by multiplying staining intensity (range 0–3) and area of positive stained tumor cells ($1 \leq 10\%$, $2 = 10–50\%$, $3 \geq 50\%$) as previously reported [15]. For statistical analyses cases were ranked by staining index, and categorized into quartiles based on frequency distribution and size of the subgroups. The upper three quartiles were combined based on similarities in survival, and defined as "ASRGL1 high". The lower quartile was defined as "ASRGL1 low", corresponding to SI: 0–2 (HPA029725) or SI: 0–1 (AMAb90907). Random TMA slides were scored by three independent observers (TF, KS and ILT) blinded for patient characteristics and outcome, and an intraclass correlation coefficient (ICC) was calculated to assess interrater reliability for each of the two antibodies. The ICC of HPA029275 score in two groups was 0.71 (95% confidence interval (CI): 0.64–0.78, n = 126 patients), while AMAb90907 score in two groups had an ICC of 0.95 (CI: 0.93–0.96, n = 105 patients). Staining and scoring of estrogen receptor α (ERα) have previously been described, defining loss of ERα as SI: 0–3 [16].

2.3. Gene expression

RNA was extracted from fresh frozen tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Samples were hybridized to Agilent Whole Human Genome Microarrays 44 k (Cat. No. G4112F) prior to scanning and normalization as previously reported [16]. In total, RNA was extracted from 237 primary tumors. For survival analyses based on mRNA levels patients were ranked by *ASRGL1* expression and divided into quartiles. The cut-off for low *ASRGL1* was defined as the lower quartile of cases.

2.4. Statistical analyses

SPSS Statistics software (version 24.0; IBM Corp., Armonk, NY, USA) was used for statistical analyses. All statistical tests were two-sided, and P-values ≤ 0.05 were considered statistically significant. ICC estimates were calculated by a single rater absolute agreement, two-way random effects model. Analyses of categorical variables were performed using the Pearson Chi-square test or Fisher's exact test, while continuous variables were evaluated using the Mann-Whitney *U* test. Disease-specific survival curves were generated using the Kaplan-Meier method, and survival between groups was compared using the log rank (Mantel-Cox) test. Time of primary surgery was used as entry date, and time to death due to endometrial carcinoma was defined as endpoint. Cox proportional hazard regression model was used to evaluate the independent prognostic impact of ASRGL1 in multivariate survival analyses.

3. Results

782 patients treated for endometrial carcinoma at Haukeland University Hospital were prospectively included in this study. Of these patients, 79% (n = 616) were classified as "ASRGL1 high" (Fig.1A) and 21% (n = 166) as "ASRGL1 low" (Fig.1B) when evaluating ASRGL1 expression in primary tumors by IHC. Validation of antibody was performed by staining 607 primary tumors with two different antibodies (AMAbb90907 and HPA029725). These antibodies had significantly correlated scoring indexes ($P < 0.001$) and similar prognostic value. The ICC estimate for AMAb90907 (ICC: 0.95, CI: 0.93–0.96) indicates excellent interrater reliability [17], and was higher than for HPA029725 (ICC: 0.71, CI: 0.64–0.68). AMAb90907 score was therefore selected for further analyses. Low ASRGL1 protein expression was significantly associated with clinicopathological characteristics of aggressive endometrial

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