POOR OUTCOME IN HYPOXIC ENDOMETRIAL CARCINOMA IS RELATED TO VASCULAR DENSITY

Running title: prognostic role of hypoxia in endometrial cancer

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ABSTRACT

BACKGROUND: Identification of endometrial carcinoma (EC) patients at high risk of recurrence is lacking. In this study, the prognostic role of hypoxia and angiogenesis was investigated in EC patients.

METHODS: Tumor slides from EC patients were stained by immunofluorescence for CAIX as hypoxic marker and CD34 for assessment of microvessel density (MVD). CAIX-expression was determined in epithelial tumor cells, with a cut-off of 1%. MVD was assessed according to the Weidner method. Correlations with disease-specific survival (DSS), disease-free survival (DFS) and distant disease-free survival (DDFS) were calculated using Kaplan-Meier curves and Cox regression analysis.

RESULTS: Sixty-three (16.4%) of 385 ECs showed positive CAIX-expression with high vascular density. These ECs had a reduced DSS compared to tumors with either hypoxia or high vascular density (log-rank p=0.002). Multivariable analysis showed that hypoxic tumors with high vascular density had a reduced DSS (hazard ratio [HR] 3.71, p=0.002), DDFS (HR 2.68, p=0.009) and a trend for reduced DFS (HR 1.87, p=0.054).

CONCLUSIONS: This study has shown that adverse outcome in hypoxic ECs is seen in the presence of high vascular density, suggesting an important role of angiogenesis in the metastatic process of hypoxic EC. Differential adjuvant treatment might be indicated for these patients.

BACKGROUND

Most endometrial carcinoma (EC) patients present with early-stage disease and have a favorable outcome. Nevertheless, 15% of all patients suffer from recurrent disease and subsequently have a poor outcome [1-3]. Approximately half of these recurrences occur in patients primarily diagnosed with low risk EC [1,4]. Improved identification of patients at high risk for recurrence is crucial to prevent both over- and undertreatment.

Hypoxia is known to be an important feature of aggressive EC and drives metastatic potential [5-8]. When solid tumors outgrow their vasculature beyond the size of 0.1 mm^3, hypoxia may occur [9]. As a response to chronic hypoxia, tumor cells will activate genes associated with more aggressive phenotype and resistance to chemotherapy and radiotherapy [10]. Hypoxia inducible factor 1 (HIF-1), formed after heterodimerization of its subunits HIF-1α and HIF-1β, plays a key role in this process [11,12]. HIF-1 activates downstream genes that enhance cell survival by maintaining intracellular pH, stimulating angiogenesis to increase oxygen delivery and switching to anaerobic glycolysis [12,13]. More specifically, an important downstream target is *carbonic anhydrase 9 (CA9)*, whose encoded protein, carbonic anhydrase IX (CAIX), regulates intracellular pH by converting carbon dioxide to carbonic acid [14]. By adaptation of tumor cells to a hostile microenvironment, tumor proliferation can commence even in hypoxic areas [15]. Also in normoxic conditions, HIF-1 can be activated, however downstream activation is present in lesser extent [16,17] In this perspective CAIX-expression, one of the key effector proteins of HIF-1, has been shown to be more specifically related to hypoxia and poor outcome [18].

Next to maintenance of intracellular pH, stimulation of angiogenesis is an important response to hypoxia. Vascular endothelial growth factor (VEGF), another downstream target of HIF-1, is also correlated with hypoxia and angiogenesis in several cancer types, including EC [19-22]. Angiogenesis can be assessed by microvessel density (MVD) and is prognostically associated

with deep myometrial invasion (MI), lymphovascular space invasion (LVSI) and poor overall survival in EC [23]. Although earlier studies suggest correlation between hypoxia, angiogenesis and poor outcome, the prognostic value has not yet been studied before [5,6]. Therefore, we have investigated the prognostic value of hypoxia and angiogenesis in EC, assessed with CAIX-expression and MVD.

METHODS

Patients

Data and tumor slides were collected previously for a study analyzing the value of L1CAM expression in ECs, which included ECs from 11 collaborating European Network for Individualized Treatment of Endometrial Cancer (ENITEC) centers [24,25]. Only cases diagnosed by an expert gynecological pathologist, with complete data on treatment and pathological examination and at least 36 months of follow-up were included. Cases with a non-endometrioid component were categorized as non-endometrioid. The 1199 cases included in the original study were randomly selected using SPSS version 22 (SPSS IBM, New York, NY) resulting in a database of 403 patients for the present study. These cases were not statistically different from the original cases for the most important baseline characteristics.

Tissue and staining

Four μm sections, derived from formalin-fixed, paraffin-embedded ECs were used to visualize CAIX and blood vessels. Sections were mounted on Superfrost slides (Menzel-Gläser). Slides were deparaffinated in Histochoise (VWR H103-4L) and rehydrated (graded ethanol: 100%-96%-70% & de-ionized water). Next, citrate buffer antigen retrieval was performed for 30 minutes (Target retrieval solution 10x pH 6 citrate, Dako Cytomation, 96 ^{o}C). Prior to incubation with the primary antibodies sections were blocked with 5% normal goat serum (Jackson ImmunoResearch) in Primary Antibody Diluent (PAD, BIORAD BUF014), 30 minutes at room temperature. Thereafter sections were co-stained for CAIX (Novus Biologicals NB100-417, 1:100) and vessels (CD34, ABCAM ab8536, 1:300), 60 minutes at 37^{0}C. Secondary incubation was performed using Cy^{TM}3 Fab Fragment Goat Anti-Rabbit IgG (H+L) polyclonal IgG (Jackson ImmunoResearch 111-167-003) for CAIX and

CF®488a Goat anti-Mouse IgG (H+L), F(ab')2 fragment polyclonal IgG (Biotium CF488A) for the vessels, 60 minutes at 37^0C. All antibodies were diluted in PAD. In between stainings, sections were rinsed with PBS (JT Baker 4391.9010). DAPI (Santa Cruz Biotechnology AB-17.0097) was used as a counterstain to stain all nuclei and finally, the sections were mounted with Fluoromount W (Serva 21634.01). Hematoxylin and eosin (H&E) staining was used for morphological evaluation. CAIX-expression was scored as the fraction of epithelial tumor cells with positive membranous staining.

Image analysis

Tumor slides were analyzed using a digital image analysis system after scanning of the whole slides with the Axio Imager D2 microscope (Carl Zeiss, GmbH, Oberkochen, Germany) using a Prior lumen 200 metal halide lamp (Prior Scientific, Rockland, USA), Axiocam 503 mono 16-bit camera (1936 x 1460 pixel, Carl Zeiss, GmbH) and a computer-controlled motorized stage (Carl Zeiss, GmbH) directed by Zen Pro software (Carl Zeiss, GmbH) [26]. Each slide was scanned for three signals: DAPI (all nuclei), Alexa488 (CD34) and Cy3 (CAIX), by means of a 10X objective using standardized shutter times for each signal (1ms, 25ms and 50ms, respectively). After scanning, grey-scale images of all three recorded signals were used for analysis.

For analysis of CAIX staining, only membranous expression on epithelial tumor cells was analyzed. Areas of necrosis, large vessels and tumor stroma, determined using hematoxylin- and eosin-stained adjacent tumor slides, were therefore manually excluded from the analysis (i-Vision for Mac; BioVision Technologies, Exton, PA, USA). Next, thresholds for segmentation of the fluorescent signals were interactively set above the background staining for each individual marker and adjusted for each sample in order to optimize the signal to background ratio using ImageJ software (Wayne Rasband, National Institute of Mental

Health, National Institutes of Health). An interactively set threshold limits inter sample variability by correction for differences in immunofluorescence staining intensity [26,27]. The resulting binary images were used to calculate the fraction of CAIX (fCAIX) relative to the total tumor area. To minimize bias of non-specific staining, only positive signals exceeding 5 pixels were included.

The MVD was measured according to the Weidner method [28]. In short, surrounding epithelial tumor cells three areas with the highest density of vessels were selected by the assessor (M.A.) using a 200X magnification. To correct for objects that exceed the image borders, only objects exceeding the left and upper border were included. To minimize bias of non-specific staining, only positive signals exceeding 2 pixels were included.

CAIX-expression was considered positive when the fCAIX was above 1% [29,30]. The MVD was dichotomized over the median. A representative example of CAIX and MVD staining is shown in **Figure 1**.

Statistical analyses

Clinicopathological differences between subgroups were compared with the χ^2 and Fisher's exact tests for categorical data and the Mann-Whitney *U*-test for continuous variables.

Kaplan-Meier curves were constructed for DSS, DFS and DDFS. The association between CAIX and MVD and disease-specific survival (DSS), disease-free survival (DFS) and distant-disease free survival (DDFS) was determined using Cox regression analysis. DSS was calculated from the date of primary treatment to the date of death caused by the disease or, for surviving patients, to the date of the last follow-up. DFS and DDFS were defined as the length of follow-up, after completion of the primary treatment, during which women survived without any clinical sign of (distant) disease recurrence. Distant recurrence included metastases in distant organs and para-aortic lymph nodes. Features identified by univariable

183 regression analysis with p<0.20, were used for multivariable regression analysis. LVSI was
184 coded as negative in case of missing data (n=108) since only substantial LVSI was recently
185 reported as relevant for prognosis of EC. If LVSI was not reported in the pathological report,
186 it was therefore assumed that LVSI was absent [31,32]. *P*-values less than 0.05 were considered
187 to indicate a significant difference. SPSS version 25 (SPSS IBM, New York, NY, USA)
188 statistical software was used to perform the statistical analyses.
189

RESULTS

Patients

After staining for CAIX and CD34, 18 of the 403 patients were excluded due to insufficient tumor tissue (n=9) and excess of non-specific background staining (n=9). Clinicopathological characteristics of the 385 patients included for analysis are shown in **Table 1**. Overall, the median age was 64 years and the median follow-up time was 58 months. Of all patients alive at the end of follow-up, 99% had a follow-up of at least 36 months. A total of 67 patients (17.4%) were diagnosed with high-grade EC, including 13 NEECs (3.4%). In total, 106 (27%) EC patients had positive CAIX-expression.Forty-seven patients (12.2%) recurred and 21 patients (5.5%) died due to the disease. Of all the patients with recurrence, 14 (3.6%) had a local recurrence, 16 (4.2%) a regional recurrence and 31 (8.1%) a distant recurrence.

CAIX-expression and microvessel density

A total of 63 carcinomas (16.4%) showed a positive membranous epithelial CAIX-expression and high degrees of vascular density, defined as a MVD above the median (**Table 1**). CAIX-expression with high vascular density was correlated with non-endometrioid histology (7.9% vs. 2.5% $p = 0.028$), but not with other clinicopathological features. Patients with CAIX-positive ECs and high vascular density experienced more recurrences (22.2% vs. 10.2% $p = 0.008$), specifically more distant recurrences (19.0% vs. 5.9% $p < 0.001$) as well as higher overall mortality (22.2% vs. 11.8%, $p = 0.027$) and EC-related mortality (17.5% vs. 3.7%, $p < 0.001$) (**Table 1**).

Figure 2 shows that CAIX-expression with high vascular density was associated with a worse DSS compared to CAIX-expression with low vascular density and negative CAIX-expression ($p = 0.002$). Interestingly, CAIX-positive ECs with low vascular density had a similar

outcome as CAIX-negative ECs. Univariable Cox regression analysis revealed that age, CAIX-expression with high vascular density, MI, FIGO-stage, grade and LVSI were significantly associated with DSS (**Figure 3**). In multivariable analysis, high age, CAIX-expression with high vascular density and tumor grade 3 remained significantly associated with reduced DSS, with CAIX&MVD as the most significant parameter (hazard ratio [HR] 3.71, 95%-CI 1.59 – 8.63, p=0.002).

Multivariable analysis showed that age, FIGO stage and LVSI were significantly associated with DFS. CAIX-expression with high vascular density was nearly significant (HR 1.87, 95%-CI 0.99 – 3.55, p = 0.054, **Figure 4**). Multivariable analysis for DDFS showed that LVSI and CAIX-expression with high vascular density were significantly associated with an reduced DDFS (CAIX&MVD: HR 2.68, 95%-CI 1.27 – 5.65, p = 0.009, **Figure 5**).

Individual contribution of CAIX and MVD

Positive CAIX-expression was associated with high tumor grade, non-endometrioid histology, higher median MVD and treatment with radiotherapy. In multivariable analysis CAIX and grade were significantly associated with DSS (HR 2.45, 95%-CI 1.05-5.73, p=0.039) (**Supplementary Table 1 and 2**). High MVD was correlated with deep MI, but not with other clinicopathological factors. In multivariable analysis, high MVD remained an independent predictor of reduced DSS (HR 2.92, 95%-CI 1.13 – 7.54, p=0.027) (**Supplementary Table 3 and 4**). Continuous scoring of CAIX-expression showed a significant correlation with DSS as well (data not shown).

DISCUSSION

In the present study we have investigated the prognostic value of angiogenesis and hypoxia, assessed with MVD and CAIX-expression. We hypothesized that angiogenesis would facilitate hematogenous spread of hypoxic tumor cells with subsequent poor clinical outcome. Additionally, we assumed that this would specifically be facilitated in hypoxic ECs, because of activation of intracellular pathways that induce an aggressive and metastatic phenotype. We have shown that CAIX-expression with high vascular density is associated with reduced disease-specific survival and distant disease-free survival. Interestingly, CAIX-positive ECs with low vascular density had a similar outcome as CAIX-negative ECs. Finally, multivariable analyses for CAIX-expression and vascular density showed that both were independent prognostic markers as well.

This is the largest study to date studying CAIX in EC. In contrast to previous studies in EC, we did find significant correlations between CAIX-expression and poor outcome, especially in case of high vascular density. Seeber *et al*. included 93 patients and found CAIX-expression in 76% of ECs [29]. In this study, no correlation between CAIX-expression and outcome was found; however small sample size and different cut-off value (all degrees of positive staining were regarded as positive) could explain why no correlation was found. Similarly, Pijnenborg *et al.* investigated CAIX-expression in 59 ECs and did not find a correlation [22]. Again, possibly this study was underpowered due to a limited sample size and low numbers of distant recurrences. Also, differences in study design (case-control study) hamper valid comparison with our results. In other cancer types, including breast carcinoma, hepatocellular carcinoma, cervical carcinoma and renal cell carcinoma, CAIX-expression is associated with poor prognosis [33-37]. More specifically, increased distant failure was seen in several solid tumor types with positive CAIX-expression [37,38].

The metastatic process is a complex step-wise process, including acquisition of a aggressive phenotype, invasion in surrounding tissues and blood vessels, survival in the circulation with subsequent extravasation and colonization in new organs [39]. Hypoxia and subsequent neoangiogenesis will intervene with several steps of this process, including promoting tumor cell survival by acquisition of a malignant phenotype and increased invasion in blood vessels [9].

A recent meta-analysis has shown that high MVD was associated with several poor prognostic variables, including deep MI, positive LVSI and poor outcome in EC, although heterogeneity due to differences in used antibodies and cut-off values hampers interpretation of these results [23]. Biologically, intratumoral neoangiogenesis in response to hypoxia will promote the formation of vasculature with high degrees of permeability and potential for rapid growth [40]. Our hypothesis that CAIX-expression with high degrees of vascular density would be associated with unfavourable prognostic features and poor outcome was based both on the facilitation of hematogenous spread in areas with high angiogenesis, and the aggressive biological behaviour of tumor cells after hypoxia [41,42]. HIF-1α is stabilized and accumulates under hypoxia, and activates transcription of numerous genes involved in angiogenesis, proliferation, and pH regulation (VEGF, CAIX, GLUT-1) [9]. Our hypothesis was supported by the fact that ECs with positive CAIX-expression and high vascular density had a decreased DSS compared to ECs with only one or none of both features. This observation supports the complex interplay underlying the metastatic processes. The observation that CAIX-positive ECs with high vascular density did not have more lymph node metastasis or local recurrences, but instead have more distant recurrences, could support the role of angiogenesis in the hematogenous rather than the lymphogenic metastatic process.

The obvious strengths of this study are the inclusion of a large and representative cohort of EC patients within the ENITEC network and the objective and reproducible measurement of

CAIX and MVD using digital imaging analyses. However there are some limitations that need to be addressed. Due to the retrospective nature of the study, there were missing values, specifically for LVSI and lymph node metastasis. Substantial LVSI is a stronger predictor for prognosis of EC compared to moderate LVSI. Also, LVSI is not routinely reported in the pathologic report at all centers. Therefore we assumed that if substantial LVSI was present it was reported and if LVSI was not reported no substantial LVSI was present [31]. Missing cases were therefore coded as negative for LVSI. Separate analyses of patients with available LVSI status did not alter the results of the primary outcome (data not shown). Another general limitation in interpretation of CAIX and MVD is the lack of standardized criteria in current literature, which hampers comparison of previous studies and this study [29,30]. However, the applied digital techniques in this study enable objective and reproducible analyses without the need for extensive pathological expertise. With the integration of digital pathology into clinical practice, comparison of future studies with our results might be easier [43,44]. Although widely used to quantify MVD, CD34 is known to also identify lymph vessels and stem cell populations, which theoretically could have led to an overestimation of our results. On the other hand, other antibodies, e.g. CD31, also carry the risk of aspecific staining. Compared to CD31, CD34-staining is known to have stronger reactivity with endothelial cells, resulting in a lower risk of staining failure [45]. Finally, generalizability to non-endometrioid subtypes can be questioned, as they comprise only 3.4% of the entire cohort. More research focused on this specific subgroup could help to strengthen these results.

This study identifies a group of patients with a poor DSS and DDFS based on CAIX and MVD. Given the increased risk of distant metastases, differential adjuvant treatment for these ECs could be explored either in the form of chemotherapy or, in the future, targeted therapies directed against angiogenesis. Because of the focal character of CAIX-expression in the tumor tissue, performing the analysis on preoperative biopsies might be challenging, but

visualization of hypoxia and angiogenesis on FDG-PET/CT scan and MRI could be an alternative, as Berg *et al.* showed recently [5].

In summary, we have found that CAIX-expression and high vascular density are prognostic markers for decreased survival in endometrial carcinoma. Combining these two markers revealed that ECs with positive CAIX-expression and high vascular density have an impaired outcome compared to ECs that have only one or none of both features. These patients experienced more distant recurrences, and therefore differential adjuvant treatment for these tumors should be explored.

ADDITIONAL INFORMATION

Ethics approval

This study was performed in accordance to the Declaration of Helsinki and was approved by the Institutional Review Board at the Radboud University Medical Centre.

Consent for publication

Not applicable

Availability of data and materials

The datasets used during the current study can be made available from the corresponding author on reasonable request.

Conflicts of interest

The author declare that they have no conflicts of interest.

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Author contribution

All authors contributed to the manuscript.

C.R., W.v.W., M.A. M.P.S., H.K., L.F.M., J.B and J.M.P. contributed to the study design.

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SUPPLEMENTARY MATERIAL

Supplementary information is available at the Britisch Journal of Cancer's website.

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FIGURE LEGENDS

Figure 1. Example of CAIX and CD34 staining in endometrial cancer. (A) Nuclear DAPI staining (blue) for visualization of tumor nuclei. (B) CAIX-staining (green) adjusted for total tumor area, meaning that only epithelial tumor cells were included in the analysis: other tissue, including stroma, necrosis, vasculature has manually been removed and is colored black by the analysis software (see 'Methods' section). (C) CD34-staining of vasculature (red) with three hotspots according to the Weidner method, marked with the interrupted lines. (D) Combined CAIX-staining and CD34-staining. Panels E, F, G and H represent representative high-magnification images of the boxed areas in A, B, C and D, respectively. Scale bar = 0.5 mm.

Figure 2. Disease-specific survival (DSS) by CAIX-expression combined with degree of angiogenesis. Log-rank test was used to compare groups.

Figure 3. Univariable and multivariable Cox regression analysis of clinicopathological parameters including CAIX combined with vascular density for DSS. The Hazard Ratios with 95%-confidence intervals are depicted by the black line. All risk factors significantly associated with DSS in univariable analysis were included in the multivariable Cox regression analysis, depicted by the grey lines.

Figure 4. Univariable and multivariable Cox regression analysis of clinicopathological parameters including CAIX combined with vascular density for disease-free survival (DFS). All risk factors significantly associated with DFS in univariable analysis were included in the multivariable Cox regression analysis, depicted by the grey lines.

Figure 5. Univariable and multivariable Cox regression analysis of clinicopathological parameters including CAIX combined with vascular density for distant disease-free survival (DDFS). All risk factors significantly associated with DDFS in univariable analysis were included in the multivariable Cox regression analysis, depicted by the grey lines.