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Expression of B7–H4 in endometrial cancer and its impact on patients' prognosis

Short title: B7–H4 in endometrial cancer

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ABSTRACT

Objectives: The aim of the study was to evaluate the B7–H4 expression in endometrial cancer cells and to investigate its relationship with patient prognosis and clinicopathological features of the disease.

Material and methods: We performed a single-center, retrospective cohort study that included endometrial cancer patients treated between 2012 and 2019. B7–H4 expression in specimens obtained from 63 patients was examined by immunohistochemical staining. The evaluation of B7H4 immunoreactivity was assessed using Immunoreactivity Scoring (IRS) system.

Results: B7–H4 reactivity was observed in all, except one, endometrial cancer patients (98%). Staining intensity: no reaction in one case, weak in 16 (24%) patients, moderate in 25 (37%), and strong in 22 (35%). Twenty-nine (46%) patients showed B7–H4 immunoreactivity in more than 60% of cells, while, in 18 (29%) cases and 16 (25%) patients, the percentages were 30–60% and < 30% respectively. Median IRS was 2 (range 0–6). We found a significantly worse overall survival (OS) rate for patients with high versus low B7–H4 IRS ($p = 0.03$), however, in multivariate analysis, the difference in patient survival was close to the

significance level (p = 0.052). B7–H4 expression was not related to histopathological type of the tumor, tumor grade, lymph node metastases, or the FIGO stage of the disease.
Conclusions: Our result suggests that B7–H4 expression might be a useful prognostic factor in endometrial cancer.
Keywords: B7–H4; endometrial cancer; IRS; overall survival; immunohistochemistry

INTRODUCTION

Endometrial cancer (EC) is the sixth most diagnosed cancer in women worldwide. Its highest incidence rate is observed in North America and Europe and the highest mortality rate is reported in Eastern Europe [1].The primary management of EC consists of surgery and/or radiotherapy, as well as systemic therapy in selected cases. Organising patients into prognostic risk groups is crucial for optimal treatment. Prognostic factors in EC are as follows: histopathological subtype, tumor grade, myometrial and cervical involvement, the presence of lymph vascular space invasion (LVSI), and the presence of lymph node or distant metastases. However, thanks to molecular profiling of endometrial cancer, more accurate predictions of patient prognosis can be achieved. Using The Cancer Genome Atlas (TCGA), four molecular subtypes (POLE ultra mutated, microsatellite instability — MSI, copy-number low, and copy-number high) of endometrial cancer were distinguished [2]. For clinical purposes, molecular profiling of endometrial cancer can be simplified and conducted by combining immunohistochemistry analysis and one molecular test (mutation analysis of exonuclease domain of POLE) [3]. However, despite the incorporation of molecular studies, the evaluation of a patient's prognosis is not always clear. This is especially in cases of MSI and copy-number low (NSMP, non-specific molecular profile) where subtypes are not always predictable [4]. Therefore, new prognostic factors in endometrial cancer are needed.

Cancerogenesis is an extremely complicated process involving many molecular and cellular mechanisms. A tumor's microenvironment influences the growth and invasion of cancer cells. The T-cells, macrophages, and natural killer cells (NK-cells) are believed to constitute a protective anti-cancer barrier and may play an important role in the antitumor immune response. However, growing tumor cells often develop a variety of mechanisms that help them escape such attention. One of these is a change in the expression of costimulatory molecules, for example members of the B7 family, resulting in impaired recognition of these cells by the host immune system [5]. B7–H4, also known as B7x, B7S1, and VTCN1, is a member of the B7 family, which downregulates the T-cell mediated response. It is responsible for activation and proliferation of T-cells, and cytokine production, and may promote tumor

growth. This was discovered in 2003 and since then has attracted the attention of many researchers due to its potential role in human diseases [6, 7]. Although the presence of B7–H4 mRNA is common in peripheral tissues, B7–H4 protein is undetectable, or expressed at low levels in normal somatic tissues, while it is usually overexpressed in inflamed tissue, viral infections, pregnancy and various types of tumor cells [6–8]. B7–H4 also occurs in a soluble form that can be detected in patients' serum [8].

In a normal endometrium, the close interaction of immune cells and hormonal changes results in the proper performance of its various functions. Obesity, hyperestrogenism and advance age, which are related to increased risk of endometrial cancer, have immunomodulatory potential. Endometrial immune cell infiltration does not only change during the menstrual cycle, but also in uterine tumors, and this phenomenon has its clinical implications [9]. Previous studies revealed that a host's immune response might be considered as an independent prognostic factor for patient survival in endometrial cancer [10]. For example, the overexpression of B7–H4 in tumor cells has been investigated as a poor prognostic factor in many neoplasms, such as cervical cancer, breast cancer, colorectal cancer, cholangiocarcinoma, and osteosarcoma [11–15]. The effects of the B7–H4 protein on the histopathological parameters and prognosis of patients with endometrial cancer are not fully understood.

Objectives

In the current study we report on the impact of B7–H4 expression in endometrial cancer cells on patients' clinicopathological parameters and survival.

MATERIAL AND METHODS

Patients

We performed a single center, retrospective cohort study that included endometrial cancer patients treated in the 2nd Department of Obstetrics and Gynecology, Centre of Postgraduate Medical Education, Warsaw, Poland between 2012 and 2019. We included only patients who underwent surgical treatment composed of hysterectomy, bilateral salpingoophorectomy and pelvic lymphadenectomy. Omentectomy was performed when serous endometrial adenocarcinoma was diagnosed. We excluded patients with distant metastases (M1), neoadjuvant chemo- or radiotherapy, patients without lymphadenectomy, and patients who also had paraaortic lymphadenectomy. Following surgical treatment, the included patients underwent adjuvant therapy according to the risk of recurrence and death. In

general, low risk patients were observed, intermediate risk patients received vaginal brachytherapy, intermediate-high risk patients received pelvic radiation, and high-risk patients were treated with a combination of both radiotherapy and chemotherapy.

The study was approved by Ethical Committee of Centre of Postgraduate Medical Education, Warsaw (84/PB/2020). Information on any patients who died was retrieved from the database of the Poland National Health System of Poland. Overall survival was calculated from the date of surgery to the date of death or the last follow-up.

Immunohistochemistry

Immunohistochemistry (IHC) analyses were performed using Anti-B7H4 antibody (ABCAM). All IHC studies were performed on 4 μm-thick sections taken from cancerous tumors fixed in 4% buffered-formalin and embedded in paraffin blocks. The specimens for IHC staining were selected according to routine histopathological protocols. Thus, among multiple tumor sections evaluated in haematoxylin and eosin (H&E) stain we selected the most representative specimen with the highest tumor volume and without necrosis. Representative images are presented in Figure 1. Evaluation of B7–H4 immunoreactivity was conducted using the Immunoreactivity Scoring system (IRS). Briefly, the evaluation included the simultaneous assessment of the number of B7–H4-positive cells and the intensity of the immunoreactivity. Staining intensity (A) was evaluated as negative or weak (0), moderate (1) and strong (2). The percentage of stained cells (B) was evaluated using the subjective method of the succeeding approximations, and the results were categorized as: 0 = no immunoreactivity or < 10% of labelled cells; 1 = 11–30% of labelled cells; 2 = 31–60% of labelled cells; 3 ≥ 60% of labelled cells. The final IRS score was calculated as a multiplication of the staining intensity and the percentage of labelled cells (A × B). The final score ranged from 0 to 6.

Statistical analysis

Non-parametric tests (Mann-Whitney or Kruskal-Wallis) were used for evaluation of median B7–H4 IRS between analysed groups. The cut off value to discriminate between low and high B7–H4 immunoreactivity was set at median IRS. High IRS was considered as equal to greater than the median IRS, while low IRS was considered as less than the median. Survival analyses were conducted using the Kaplan-Meier survival curves and the differences in patient survival were compared using the log-rank test. Multivariate survival analysis was conducted using Cox proportional-hazards regression with the backward method of variable

entry. We used the following confounders for model development: lymph node metastases, tumor grade, tumor stage, high B7–H4 IRS. Statistical analysis was conducted using MedCalc 11.4.2.0., MedCalc Software, Seoul, Republic of Korea, and GraphPad InStat 3.06, GraphPad Software Inc., San Diego, CA, USA.

RESULTS

Patients' characteristics

We identified 63 patients who met the inclusion criteria. The median patient age was 67 and the range from 47 to 92 years. The study group included 47 patients with low grade endometrioid endometrial carcinomas, 10 with high grade endometrioid endometrial cancers, 3 with serous, and 3 with clear cell endometrial carcinomas. The TNM classification of the T of the tumors was as follows: T1a — 16 patients; T1b — 29 patients; T2 — 12 patients; T3a — 1patients; T3b — 4 patients; and T4 — 1 patient. The median follow-up period for patients was 5 years. The median number of harvested lymph nodes was 15 (interquartile range, IQR = 8 – 20). Lymph node metastases were found in 9 (14%) patients.

B7–H4 immunoreactivity

B7–H4 reactivity was observed in all, except one, endometrial cancer patients (98%). Positive apical membranous expression was observed in 14 cases (22%), cytoplasmatic and membranous expression in 37 cases (59%) and both in 11 cases (17%). Staining intensity was as follows: no reaction in 1 case, weak in 16 (24%) patients, moderate in 25 (37%) patients, and strong in 22 (35%) patients. Twenty-nine (46%) patients presented B7–H4 immunoreactivity in more than 60% of cells, while, in the case of 18 (29%) and 16 (25%) patients, the percentage of labelled cells were 30–60% and < 30%, respectively. Median IRS was 2 (range 0–6). High IRS for B7 H4 was found in 41 patients (65%), while low B7–H4 IRS was observed in 22 (35%) patients. We did not find any relationship between B7–H4 IRS and the histopathological type of the tumor, lymph node metastases, depth of myometrial invasion, or FIGO stage of the disease. Detailed results are summarized in Table 1.

Survival analyses

We found significantly worse OS of patients with high versus low B7–H4 IRS ($p = 0.03$). In the patient group with high B7–H4 IRS, the median OS was 60 months (range 2–92 months), while in the group with low B7–H4 IRS the median OS was not reached (range 34–91 months). Five-year OS was 69% and 92% in the group of patients with high and low B7–

H4 IRS respectively. Survival curves are presented in Figure 2A. In the multivariate, adjusted, survival analysis, we found that the presence of lymph node metastases [Hazard Ratio (HR) = 2.9; 95% confidence interval (CI) 1.03–8.16, p = 0.045], tumor stage (HR = 3.14; 95% CI 1.79–5.49; p < 0.01) were independently related with shortened overall survival. The association between high B7–H4 IRS and shortened patient survival approached the level of significance (HR = 4.43; 95% CI 0.99–19.76; p = 0.052). In our cohort, tumor grade was not associated with patient survival. We found no difference in patient's survival in relation to the type of B7–H4 immunoreactivity (p = 0.87, Fig. 2B). Across all three groupings, the median OS was not reached. The range of survival was as follows: apical membranous expression 3–89 months, cytoplasmatic and membranous expression 2–88 months, and in cases with both type of expression 37–92 months. Five-year OS were 86%, 74% and 91% respectively.

DISCUSSION

B7–H4 is excessively produced in many tumors, including breast cancer, cervical cancer, and some types of ovarian cancers [15–17]. However, data regarding B7–H4 expression in endometrial cancer are sparse and those studies that have been reported are consistent with each other. Miyatake et al. [18], reported B7–H4 immunoreactivity in all of the specimens of normal and hyperplastic endometrium as well as in malignant cells, nevertheless the intensity and percentage of B7–H4 staining in hyperplastic to malignant endometrial cells was higher than for a normal endometrium. Also, in the study of Qian et al., the intensity of reaction in normal cells was less intense when compared to cancer cells [19]. These findings are not consistent with those obtained by Liu et al. [20], who also reported B7–H4 immunoreactivity in all of the normal and malignant specimens, however, there were no differences in the expression levels of B7–H4 between endometrial cancer and normal endometrium, with both classified as high. Similarly, Vanderstraeten et al. [21], detected B7–H4 in all cases of normal endometrium and primary endometrial carcinoma, as well as in recurrent endometrial carcinoma, and in 90% of metastatic endometrial carcinoma. These results were consistent with our study, while we found that B7–H4 was expressed in 98% of endometrial cancers and moderate or strong expression was observed in most patients. Similarly, Bregar et al. [22], observed a prevalence of B7–H4 in endometrial cancer cell. The incidence of B7–H4 expression in our study and in those previously mentioned was higher than that reported in the study by Zong et al., where B7–H4 immunoreactivity only occurred in 71.5% of endometrial cancers [23].

B7–H4 is a transmembrane protein, but its presence in the intracellular compartment has also been reported in some tumors [24, 25]. Membrane B7–H4 may be responsible for tumor progression as an effect of suppressing T-cell immunity, while intracellular B7–H4 probably does not have such a function though its precise role is not well known [24]. However, it was shown that intracellular B7–H4 promotes cell proliferation, adhesion and invasion [26]. It may also suppress apoptosis [8]. Membranous B7–H4 has an impact on decreased density of tumor infiltrating lymphocytes (TILs), whereas intracellular B7–H4 has no such effect [27]. A high number of TILs seems to be related with improved prognosis in cancer patients [10]. In our study we observed cytoplasmic and membranous reaction in the main. Similarly in the other studies, B7–H4 expression in endometrial cancer is described as cytoplasmic and circumferential membranous, whereas a predominantly apical reaction was mostly detected in normal endometrial tissue [18–20]. In our study, we found no difference in either patient prognosis or clinicopathological features of the tumors regarding different locations of B7–H4 immunoreactivity. Therefore, the biological significance of different sites of B7–H4 immunoreactivity requires further research.

Previous studies including patients suffering from colorectal, gastric, lung, prostate, and thyroid cancers, and melanoma and osteosarcoma reported that high expression of B7–H4 was correlated with the tumor's depth of invasion, distant metastasis, cancer progression, higher recurrence rate, or poorer patient outcome [14, 25, 28–30]. In the present study we analysed the expression of B7–H4 in endometrial cancer cells and its impact on patient survival, histopathological type of the tumor, lymph node metastases, depth of myometrial invasion, and FIGO stage of the disease. We observed a significantly worse prognosis of patients with high B7–H4 immunoreactivity when compared to patients with low B7–H4 immunoreactivity. However, in multivariate analysis, when other risk factors were included, the significance of B7–H4 was only borderline ($p = 0.052$). In contrast to our result, Miyake et al., did not observe any impact from the percentage of positive cells and the intensity of staining on disease free survival [18]. Furthermore, in the study by Zong et al., the authors even found improved patient survival in cases of endometrial cancer with B7–H4 expression. That association was significant both in the whole study group, and in the NSMP subtype. However, they observed a more favourable histopathological profile (low grade, stage I of the disease, and endometrioid histology) in B7–H4 expressing endometrial cancers [23]. On the contrary, in our study, we found no relationship between B7–H4 IRS and the histopathological type of the tumor, lymph node metastases, depth of myometrial invasion, or FIGO stage of the disease. Additionally, the grade of the tumor (high vs low) was not associated with B7–H4

expression. Similar results were also obtained by Bregar et al., whose authors found no relationship between B7–H4 expression and tumor grade and histopathological type. Furthermore, they reported that B7–H4 expression was independent of MSI status [22].

Tumors classified as MSI are usually insensitive to chemotherapy, however they produce many antigens because of a huge number of mutations. High tumor mutation burden result in the expression of numerous tumor antigens, and therefore, these tumors are more prone to immunotherapy [31]. PD-1, a receptor for PD-L1 (B7–H1) was shown to be a good target for immunotherapy in endometrial cancer [32]. Recently the FDA (Food and Drug Administration) approved pembrolizumab and dostarlimab, both anti-PD-1 antibodies, for therapy of advanced endometrial cancer that is MSI-H [33, 34]. Considering high B7–H4 expression in endometrial cancers, this immune checkpoint inhibitor also seems to be a promising target for immunotherapy in endometrial cancer, and some clinical trials are currently in progress [8, 32].

In summary, there are many conflicting data on B7–H4 expression in endometrial cancer. It may be due to the retrospective character of available studies and the subjectivity of immunohistochemical evaluation. In addition, in all the research mentioned has differed regarding antibodies against B7–H4, scoring systems, and cut off values, all of which might have influenced the results of the studies. The same problems are reported in the evaluation of PD-L1 expression as a predictive biomarker for sensitivity to immune checkpoint blockade.

Similarly, the main limitation of our study is its retrospective character and usage of subjective immunoreactivity assessment. Additionally, as we mentioned above, there is no any universal B7–H4 immunoreactivity scoring system available in the literature, so we just decided to use one of previously used. Therefore, this problem requires further research [35].

CONCLUSIONS

Our result suggests that B7–H4 expression might be a useful prognostic factor in endometrial cancer.

Article information and declarations

Data availability statement

Available on request.

Ethics statement

The study was approved by Ethical Committee of Centre of Postgraduate Medical Education, Warsaw (84/PB/2020).

Author contributions

Katarzyna Gorzelnik — 50% contribution, concept and article writing.

Anna Wasążnik-Jędras — 15% contribution, article writing, IHC analysis.

Łukasz Wicherek — 15% contribution, concept and article revision.

Sebastian Szubert — 20% — statistical analysis, writing.

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Conflict of interest

The authors assert they have no conflicts of interest to declare.

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Table 1. Relationship between the B7–H4 immunoreactivity score (IRS) and clinicopathological characteristics of endometrial cancer patients

	Median B7–H4 IRS	Range	p-value
Histopathological type			
Endometrioid low-grade (n = 47)	2.0	0–6	p = 0.28
Endometrioid high-grade (n = 10)	3.5	0–6	
Non-endometrioid (n = 6)	0	0–6	
Lymph node metastases			
No (n = 54)	3.0	0–6	p = 0.45
Yes (n = 9)	2.0	0–6	
Myometrial invasion			
Below 50% (T1a; n = 16)	3.0	0–6	p = 0.31
Above 50% (T1b; n = 29)	3.0	0–6	
FIGO stage of the disease			
I (n = 45)	3.0	0–6	p = 0.59
II (n = 12)	2.0	0–6	
III and IV (n = 6)	3.0	0–6	

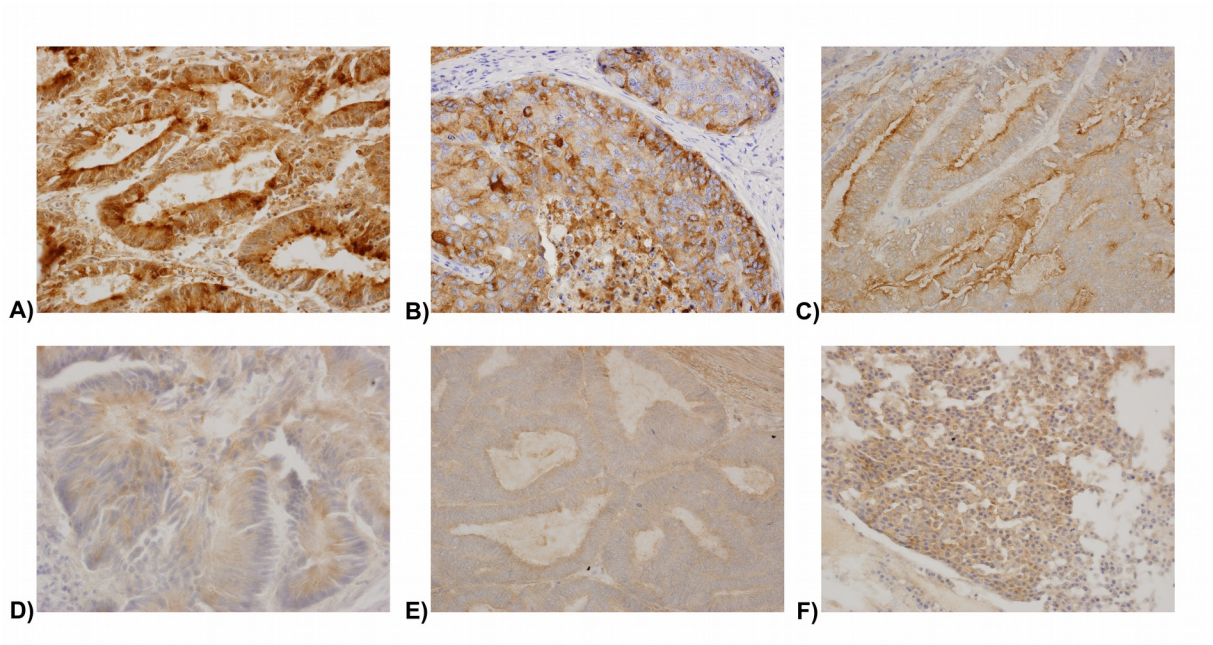

Figure 1. Representative microphotographs of B7–H4 immunoreactivity in endometrial cancer. **A.** Endometrioid endometrial carcinoma with strong apical and cytoplasmatic B7–H4

expression; **B.** Endometrioid endometrial carcinoma with strong cytoplasmatic membranous B7–H4 expression; **C.** Endometrioid endometrial carcinoma with weak cytoplasmatic and membranous B7–H4 expression; **D.** Endometrioid endometrial carcinoma with weak apical B7–H4 expression; **E.** Endometrioid endometrial carcinoma with weak apical B7–H4 expression; **F.** Serous endometrial carcinoma with moderate cytoplasmatic and membranous B7–H4 expression

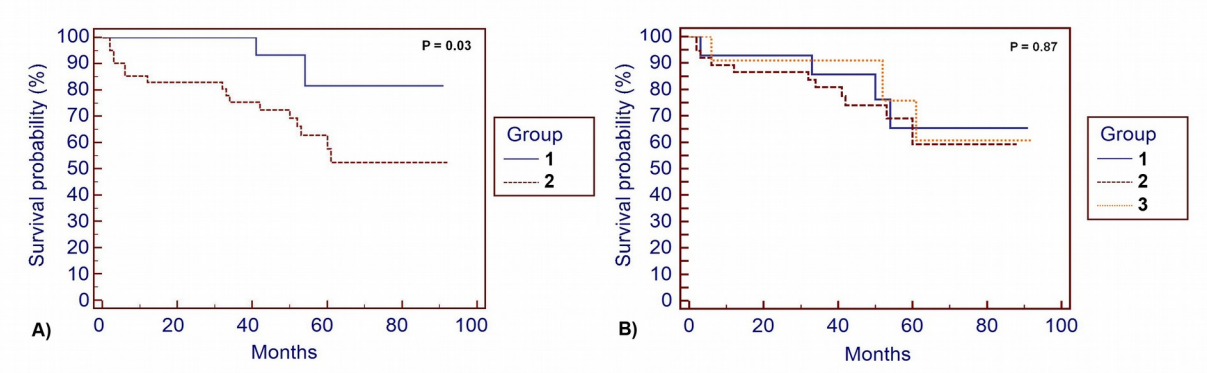

Figure 2. Survival curves of endometrial cancer patients according to B7H4 immunoreactivity. **A.** Survival curves of patients with low and high B7–H4 immunoreactivity score (IRS). Group 1. Low B7–H4 IRS, median overall survival (OS) was not reach, 5-year OS 92%. Group 2. High B7–H4 IRS, median OS — 60 months, 5-year OS 69%, p = 0.03; **B.** Survival curves according to the type of B7H4 immunoreactivity. Group 1. apical membranous expression, mOS — not reached, range: 3–89 months, 5-year OS was 86%. Group 2: cytoplasmatic and membranous expression, mOS — not reached, range: 2–88 months, 5-year OS was 74%. Group 3: both type of expression, mOS — not reached, range: 37–92 months, 5-year OS was 91%. p = 0.87