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ORIGINAL ARTICLE

Expression of hPEBP4 negatively correlates with estrogen and progesterone receptors in endometrial carcinoma

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Summary

Purpose: *To investigate the expression of human phosphatidylethanolamine-binding protein 4 (hPEBP4) in endometrial carcinoma and its relation with progesterone receptor (PR) and estrogen receptor (ER).*

Methods: *Forty-five samples of endometrioid endometrial carcinoma (EEC), 12 samples of atypical endometrial hyperplasia, and 30 samples of normal endometrium were examined. Samples were studied by immunohistochemistry for PR, ER and hPEBP4 expression. Expressions were statistically quantified and analyzed.*

Results: *Expressions of PR and ER were significantly higher in normal endometrium than in cancer. Expression of hPEBP4 was significantly lower in normal endometrium. The expression of hPEBP4 was significantly higher in advanced-stage endometrial cancer, whilst higher but insignificant trend was noticed in higher grade carcinoma. Statistically insignificant trend of negative ER and PR expression with higher grade or stage was noticed. The expression of hPEBP4 was negatively correlated to ER and PR in EEC.*

Conclusion: *The expression pattern of hPEBP4 indicated that hPEBP4 interacted with ER and PR in EEC and could thus become a possible target for the development of novel treatment against this malignancy.*

Key words: *endometrial carcinoma, estrogen receptor, human phosphatidylethanolamine-binding protein 4, progesterone receptor*

Introduction

Endometrial carcinoma is the most common malignancy of the female genital tract in the Western world and the second most common female genital malignancy in China. EEC accounts for up to 80% of this malignancy and is the most common subtype with a recurrence rate from 15 to 20%. The serous subtype on the other hand, accounts for approximately 10% of all cases, but is responsible for more than 50% of the recurrences [1]. Since recurrent disease carries a poor outcome, surgical removal is rarely used in this setting. Treatment modalities for recurrent cases comprise irradiation or systemic therapy (chemotherapy or targeted therapy). During the past several decades, molecular events that are involved in human tumorigenesis have been intensively investigated for novel treatments of cancer. Definitions and research of signalling pathways regulating cell growth, cell cycle, and programmed cell death, indicated numerous targets for novel anticancer agents. Albeit traditional therapies, like chemotherapy, still cover the majority of therapeutic options in a variety of malignacies, targeted therapies have emerged for some cancers which showed promising activity in prolonging survival for advanced-stage and recurrent patients [2]. EEC is closely related to steroid hormone receptors including ER and PR. Targeting these receptors is thus a logical option for a subset of receptor-positive endometrial cancers. Low-grade EEC featuring a long disease-free interval is most likely to be responsive to hormonal agents [3].

hPEBP4 is a novel member of human phosphatidylethanolamine-binding protein (hPEBP) family. It has been reported that hPEBP4 not only inhibits the mitogen-activated protein kinase

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(MAPK) signalling pathway [4-6] but also inhibits apoptosis [7,8]. Recent research has demonstrated that high expression of hPEBP4 was related to tumorigenesis, invasion and metastasis in a variety of tumors including breast, ovarian, prostate, colorectal, and lung cancers [9-12]. hPEBP4 is believed to inhibit proteasome-dependent ER degradation through the Src pathway, thus enhancing ER-mediated transactivation and promoting the proliferation of breast cancer cells in response to estrogens [13]. Nonetheless, hPEBP4 has not yet been studied in endometrial cancer which is closely related to ER. We have thus conducted the current study aiming at demonstrating the expression of hPEBP4 in endometrial cancer, investigating its association with ER and PR, and analyzing the possible mechanisms therein.

Methods

Specimens

From January 2011 to April 2012, 45 samples of EEC, 12 samples of endometrial atypical hyperplasia, and 30 samples of normal endometrium were collected from the Department of Gynaecology and Obstetrics, Gynaecology Hospital of Fudan University. Cancer and atypical hyperplasia samples were obtained from surgical material and normal control samples were acquired from curettage. The age of patients ranged from 31 to 72 years (median 53.6). All specimens were stained by haematoxylin & eosin (H&E) for confirmation of diagnosis, grade and stage by 2 independent pathologists. Informed consent was obtained from all patients and this study was approved by the local ethics committee.

Immunohistochemistry and assessment

All samples were formalin-fixed and paraffin-embedded. Slices were cut at 4 μm and mounted on polylysine-coated glass slides. Endogenous peroxidase of deparaffinized sections was blocked through incubation with 3% hydrogen peroxide for 15 min. After washing with PBS (phosphate buffer solution), all samples were fixed with citrate buffer (0.01 M, pH 6.0) and sealed for 10 min at room temperature with 5% normal goat serum to block non-specific binding. A hPEBP4 goat anti-human polyclonal antibody (Abcam, UK; 1:200 dilution) was added and incubated at 4°C overnight. Following wash with PBS, the biotinylated secondary antibody (polyclonal biotin-conjugated donkey anti-goat IgG; Abcam, UK; 1:200 dilution) was added and incubated at 37°C for 15 min. After wash with PBS, a working solution of HRP-conjugated streptavidin was added. Following the last wash with PBS, DAB (diaminobenzidine tetrahydrochloride) was used for coloring, and the samples were counterstained with haematoxylin, dehydrated, cleared, and mounted with neutral gum. For positive control, we used paraffinized sections of breast cancer. For negative control, we applied PBS in the place of the primary antibody. Immunohistochemical procedures of ER and PR were similar. The reagents we used were both monoclonal antimouse antibodies (Dako Cytomation, Copenhagen, Denmark). Both antibodies were buffered at pH 6.0 and were color-developed by En Vision chainpolymer method (Dako).

Samples were processed by two experienced pathologists using the double-blind method by randomly selecting 5 high-power (×200) fields. The immunohistochemical staining resulting from each field was analyzed semiquantitatively by the sum or product of intensity and extensity (the proportion of the cells stained under a microscopic field). For hPEBP4, the extensity was graded as follows: 0 for 0-10% of tumor cells stained; 1 for 11-25% of cells stained; 2 for 26-50% of cells stained; and 3 for >50% of cells stained. Intensity of staining was graded as follows: 1 for light yellow; 2 for dark yellow; and 3 for brown. The final value of each slide was obtained from the sum of these two factors as follows: 0 for negative (1-2), 1 for mild (3), 2 for moderate (4), and 3 for strong (5-6). For both ER and PR, staining intensity was graded from 0 (no staining) to 3 (strong staining). The extensity of immunopositive cells was graded as 0 (no tumor cell positive), 1 (positive staining in < 10% of the tumor cells), 2 (positive staining in 10-50% of the tumor cells), or 3 (positive staining in >50% of the tumor cells). A staining index was calculated as the product of staining intensity and the extensity (score 0-9). The staining indices were clustered as follows: 0 = 0, 1 = 1- 3, 2 = 4-6, and 3 = 9.

Statistics

The SPSS 17.0 for Windows software was used for statistical analyses. All data were presented as mean ± standard deviation (SD). The Mann-Whitney *U*-test was used for comparing scores of PR, ER and hPEBP4 between 2 categories. The Kruskal-Wallis test was applied for comparisons of scores between 3 or more categories. Correlations between the expression of PR, ER and hPEBP4 in terms of scores were evaluated with the Spearman's correlation test. A p-value of < 0.05 was accepted as statistically significant.

Results

Immunopositive areas for PR (Figure 1A-C) and ER (Figure 1D-F) were localized within the cell nuclei, whilst for hPEBP4 they were within the cytoplasm (Figure 1G-I). Significant expression differences between normal endometrium and cancer tissue was found for ER, PR and hPEBP4 ($p<0.0001$ for each group, respectively). Nonetheless, when the expression between normal endometrium and atypical hyperplasia was investigated and compared, a significant loss in PR and ER expression was found in atypical hyperplasia ($p=0.0210$ for PR and $p=0.0289$ for ER), whilst no change was noticed in hPEBP4

Table 1. Expression of PR, ER and hPEBP4 in association with clinicopathological parameters in endometrial cancer (mean ± standard deviation)

Parameters	*N*	*PR*	*ER*	*hPEBP4*
Normal	30	2.0 ± 0.9	1.9 ± 0.9	1.0 ± 0.7
p-value*		0.0210	0.0289	0.9017
Atypical hyperplasia	12	1.3 ± 0.7	1.3 ± 0.7	1.0 ± 0.7
p-value§		0.3779	0.2312	0.0020
Carcinoma	45	1.0 ± 0.7	1.2 ± 0.8	1.9 ± 0.8
p-value†		< 0.0001	< 0.0001	< 0.0001
Tumor grade				
G1	27	1.1 ± 0.8	1.3 ± 0.8	1.7 ± 0.7
G2	11	0.8 ± 0.8	1.0 ± 0.7	2.1 ± 0.8
G3	7	1.1 ± 0.7	0.9 ± 0.7	2.4 ± 0.8
p-value		0.5038	0.3418	0.0593
Tumor stage				
Ia	17	1.2 ± 0.8	1.5 ± 0.9	1.5 ± 0.7
Ib	14	0.9 ± 0.8	1.1 ± 0.7	2.1 ± 0.6
II	8	0.9 ± 0.6	0.8 ± 0.5	2.3 ± 0.9
III	6	1.2 ± 0.8	0.8 ± 0.8	2.3 ± 0.8
p-value		0.4481	0.1209	0.0247

*Comparison between normal endometrium and atypical hyperplasia
§Comparison between atypical hyperplasia and carcinoma
†Comparison between normal endometrium and carcinoma
PR:progesterone receptor, ER:estrogen receptor, hPEBP4:human phosphatidylethanolamine-binding protein 4

(Table 1). Likewise, the expression of hPEBP4 increased significantly in the cancer group compared to hyperplasia (p=0.0020), whilst ER and PR remained unchanged between the groups (Table 1). Concerning tumor grade category, the expression of PR and ER remained unchanged with progressing grade (Table 1). hPEBP4, however, showed a trend for higher expression with higher tumor grade with a p value close to statistical significance (Table 1). Expression of hPEBP4 was significantly higher with progressing stage (p=0.0247). On the contrary, the expression of ER and PR did not change significantly within different stages (Table 1).

When correlations were investigated, a significant negative correlation between the expression of PR and hPEBP4 was noticed in the EEC group (p=0.027; *r*=-0.329). Significant negative correlation between ER and hPEBP4 was also revealed in EEC (p=0.013; *r*=-0.367). There was, however, no significant correlation between PR and ER in the EEC group (p=0.480; *r*=108).

Discussion

Endometrium is a principal target tissue of the pituitary–gonadal axis. It is one of the most dynamic tissues in humans, undergoing proliferative and secretory changes that are thought to be primarily controlled and mediated by the steroid hormones. These hormones in human endometrium act via the ER and PR [14]. The pattern of ER and PR expression as well as the ratio of the expression levels of the 2 factors may play a critical role in normal endometrial function and pathogenesis. Expression and the relationship of these two hormones can have essential clinical implications [15]. ER and PR belong to the steroid hormone receptor superfamily that comprises estrogen, progesterone, androgen, glucocorticoid and mineral corticoid receptors. Binding of a ligand to these steroid receptors leads to a conformational change in their structure with subsequent dimerization. Apart from the well known ERα, there is a recently found, second genetically distinct ER. This novel receptor, ERβ, is highly homologous to the classical one (ERα), which can bind estradiol with high affinity and stimulate transcription of an ER target gene [16]. However, ER knock-out studies show that the predominant expression of ERα resides in the uterus, being the major mediator of estrogen action. For ERα, presence of various proteins has been reported in normal endometrium, endometrial hyperplasia and carcinomas. Progesterone acting through PR is the physiological negative regulator

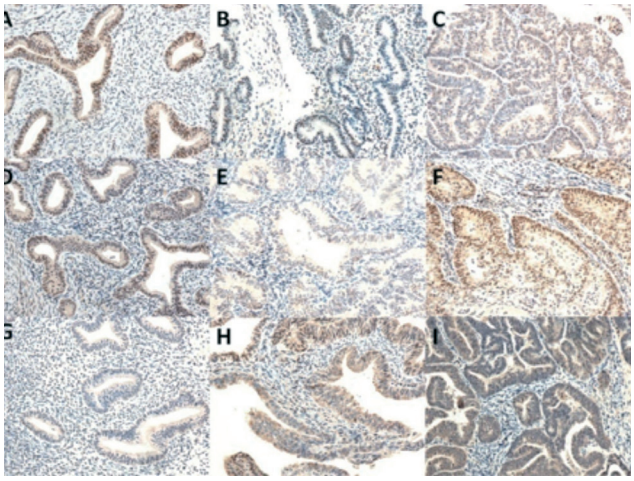

Figure 1. Immunohistochemical staining of progesterone receptor (PR) (A-C), estrogen receptor (ER) (D-F), and human phosphatidylethanolamine-binding protein 4 (hPEBP4) (G-I) in normal endometrium, atypical endometrial hyperplasia, and endometrial cancer, respectively.

of estrogen action in the endometrium. PR has two major isoforms, PR-A and PR-B. It is believed that the main function of progesterone-activated PR-A in the endometrium is to downregulate estrogen activity by preventing ERα from transactivation [17].

It has long been believed that steroid receptor proteins were localized in the cytoplasm, and therefore the standard biochemical assay procedures for assessment of ER and PR in breast cancer were the dextran-coated charcoal (DCC) and the sucrose gradient assay. Nonetheless, an immunohistochemical study of target tissue with novel antibodies against ER unequivocally displayed only nuclear immunopositive activity [18]. Furthermore, a study of enucleated rat pituitary cells demonstrated that the majority of ER locates in the nucleus, with little or none being localized in the cytoplasm [19]. Obviously, biochemical processing for these receptors results in diffusion of display from the nucleus to cytoplasm. Cyclical sex steroid expression is related to the known alterations of their receptors' immunolocalization in the endometrium. ER concentrations are maximal in the proliferative phase and decline in the secretory phase in both the glandular and stromal compartment. Mylonas et al. suggest that ERβ in the endometrium predominantly emerges during the proliferative phase and declines when the menstruation continues [20]. A disrupted expression of ERα and β may play critical roles in endometrial pathogenesis and carcinogenesis [21]. Whether ERβ expression is influenced by PR subtypes or progesterone remains unclarified. In contrast, no significant variations in PR content or distribution throughout the menstrual cycle have been observed. A higher PR-B concentration during the proliferative and early secretory phase, decreasing significantly in the late secretory phase, has been shown by using a monoclonal antibody from clone SAN27/Mouse Ig (Novocastra, Newcastle, UK) [22].

Endometrial cancer is the most common gynaecologic malignancy in the Western world and occurs in women both during their reproductive years and postmenopausally. It mostly occurs in females with excessive estrogens, such as estrogen-only hormone replacement therapy (HRT), obesity, polycystic ovary disease, nulliparity, estrogen-producing tumors and anovulation [23]. Endometrial cancer can originate from hyperplastic lesions. This reflects the failure of current diagnostics to identify premalignant lesions and endometrial cancer patients with poor prognosis. Therefore, immunohistochemistry to detect different specific markers can be a promising alternative to screen for high-risk patients [15]. It is believed that ER and PR are independent prognostic factors for endometrial carcinoma. PR is thought to be a more predictive factor of disease-free survival, whilst some authors also report that steroid receptors do not constitute independent prognostic factors for endometrial cancer [24]. ERα is believed to give prognostic information independent of tumor stage and grade in patients with endometrial cancer. PR-A counters estrogen action directly by inhibiting ER function in a dominant-negative way and a decrease in PR-B has been observed in poorly differentiated endometrial cancer cell lines. There is also a study indicating that PR-A is more predictive than ER concerning disease-free survival in endometrial cancer [25]. Nevertheless, several conflicting results report that loss of ER but not PR is not associated with poorer survival, resulting in controversial discussions with regard to the usefulness of the determination of these receptors in endometrial cancer patients.

hPEBP4 is a novel member of the PEBP family. Current studies have shown that hPEBP4 is an anti-apoptosis protein and is highly expressed in a variety of tumors [8]. Studies show that hPEBP4 is preferentially expressed in estrogen-related tumors. For instance, hPEBP4 is highly expressed in MCF-7 breast cancer cells. hPEBP4 increases the transcriptional activity of ERα by inhibiting of ERα proteasomal degradation which is independent from the regulation of ERK1/2 and Akt phosphorylation [13]. hPEBP4 can also promote breast cancer cell metastasis to the lung [26]. Expression levels of hPEBP4 in ovarian and prostate cancer are higher compared to normal tissues. In normal tissues, hPEBP4 co-localizes with lysosomes. hPEBP4 translocates to the cell membrane and interacts with Raf-1 and MEK1 to inhibit the MAPK signaling pathway by stimulat-

ing TNF-α [8]. Overexpression of hPEBP4 inhibits TNF-α-induced apoptosis in L929 fibroblast cells [7,27] and protects CaoV-3 ovarian cancer cells from TRAIL-induced apoptosis [12]. Recent studies also show that the specific expression of PEBP4 in retinal ganglion cells promotes cell migration. In all, hPEBP4 may play an important role in the incidence and development of tumors.

Given the aforementioned facts, we have investigated for the first time the expression of hPEBP4 in human EEC. Similar to the reports concerning hPEBP4, we have confirmed that this is a pro-tumorigenic factor in endometrial cancer. Overexpression of hPEBP4 may lead to cancer development from normal endometrium. Nonetheless, the role of hPEBP4 may emerge in a later stage of carcinogenesis since the expression does not change in the transition between atypical hyperplasia and normal tissue but between the hyperplasia and cancer. This pattern was different from ER and PR in our series. It is, however, interesting that we showed no correlation either in ER or in PR and tumor stage or grade. Only trend of loss of expression of both receptors with progressing stage or grade was noticed. This ambiguity somehow conforms to the dispute in the current literature concerning PR and ER in normal or malignant endometrium. However, we found that expression of hPEBP4 negatively correlates with both receptors. This finding lends confidence that this pro-tumorigenic factor may interact with PR and ER via a pathway where novel targeted therapy could be aimed at.

Our study has some limitations. The amount of samples in the current study was limited and the number should be extended to confirm the results. The antibodies we applied in the current study for PR and ER recognized the major type of both receptors, namely ERα and PR-A. Whether these subtypes are also involved in this correlation pattern should be a matter of further investigation.

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