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ARID1A and GPR30 expression patterns characterize the different histological subtypes of endometrial carcinoma

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Objectives: It is of utmost importance to investigate the newly discovered immunohistochemical proteins that are helpful in differentiating various histological subtypes of endometrial carcinomas (ECs). In this study, we aimed to compare the localization and expression profile of selected proteins (ARID1A, nidogen 2 (*NID2*), LRH-1, and GPR30) in 60 early-staged (I and II) G2/G3 ECs with different histological subtypes. *Methods*: Endometrioid-type (n = 20), serous (n = 20), and clear-cell (n = 20) ECs were immunohistochemically stained applying the anti-ARID1A, -*NID2*, -LRH-1, and -GPR30 antibodies. Normal endometrial samples (n = 8) were selected as a control group. *Results*: In general, 95% (19 out of 20) and 100% (20 out of 20) of endometrioid and serous samples revealed moderately/intense cytoplasmic/nuclear ARID1A immune-positivity, while only four out of 20 (20%) clear-cell carcinomas showed moderate staining. A significant difference in ARID1A expression was noted between different histological subtypes of ECs (clear-cell cancer vs endometrioid cancer, $p < 0.001$, and clear-cell cancer vs serous cancer, $p < 0.001$). Cytoplasmic *NID2* staining did not differ significantly between histological subtypes. Most of the endometrioid (19/20; 95%) and serous (19/20, 95%) neoplasms revealed intense cytoplasmic LRH1-immunopositivity. Weak/moderate GPR30 cytoplasmic reactivity was detected in 16 out of 20 (80%) endometrioid EC, however, this protein was neither noted in clear-cell and serous neoplasms nor normal endometria. Differences in GPR30 immunoreactivity between endometrioid cancer and serous/clear-cell cancer were of significant values ($p < 0.005$). *Conclusion*: Weak ARID1A expression may be associated with gene alterations in selected EC histological subtypes. GPR30 staining may help to differentiate various histological EC subtypes.

Keywords

Endometrial cancer; Immunohistochemistry; ARID1A; Nidogen 2; LRH-1; GPR30

1. Introduction

Endometrial cancer (EC) is the most common female genital cancer in developed countries and the second most common gynecological neoplasm worldwide [1, 2]. The incidence rate of EC has been reported to have an increasing trend worldwide. In Poland, 5984 new cases were diagnosed in 2017, and 1761 women died from the disease [3]. Over the last few decades, a population-wide increase in EC morbidity has also been reported [2]. In general, EC is most often diagnosed in women aged 50–70 years, although about 5% of the patients developed EC before 40 years of age [4].

Consequently, it is of utmost importance to investigate the newly discovered immunohistochemical markers that help to differentiate various histological subtypes of EC [5, 6]. Interestingly, recently discovered proteins—ARID1A, *NID2*, LRH-1, and GPR30—are known to be expressed in various human neoplasms, including endometrial and ovarian carcinomas [7, 8]. However, there is a limited number of data assessing the role of these proteins in different histological subtypes of ECs (PubMed©).

ARID1A, a member of the SW1/SNF chromatin remodeling complex, is altered in endometrioid-type (EEC) and clear-cell (CC) carcinomas of the ovary and endometrium [8–12]. A recent study revealed concordance results between the mutational status of the gene and IHC results [8]. They finally assumed that "this will be useful for recruiting patients for clinical trials based on *ARID1A* mutational status" [8].

The protein, produced by fibroblasts, encoded by the *NID2* is involved in maintaining the basement membrane structure [13–15]. This intracellular matrix protein controls a number of intracellular processes, including cell adhesion, migration, differentiation, and apoptosis [15]. *NID2* connects the laminin and collagen IV networks, stabilizing the structure of the basement membrane, and is involved in cell-adhesion processes. Moreover, it establishes contraction with cellular integrin proteins [15]. Interestingly, *NID2* significantly inhibits the development of cancer and metastases [16–19].

Liver receptor homolog-1 (LRH-1) has been reported to be altered in a variety of human malignancies [20–22]. It promotes tumor cell proliferation and the development of distant metastases [23, 24]. Additionally, it is involved in the cell-cycle progression and apoptosis [21]. LRH-1 expression was significantly associated with clinical and pathological stage, depth of invasion, and lymph node metastases in colon cancer patients [22]. EC cells express the nuclear receptor LRH-1 at the mRNA and protein levels, and activate the genes, encod-

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ing the steroidogenic enzymes involved in estradiol synthesis [25]. The nuclear receptor LRH-1 is a well-known regulator of steroidogenic gene expression in normal gonadal and adrenal cells [25].

G protein-coupled estrogen receptor (GPR30), described in 2005, binds to estradiol and is responsible for rapid estradiol activity in different human cells [26, 27]. The effects of GPR30 include activation of MAPK and PI3K signaling pathways, stimulation of adenylyl cyclase, and upregulation of FOS and CTGF [26]. GPR30 regulates fundamental biological processes, such as tumor growth and homeostasis, and is also involved in tumor initiation and progression [27, 28]. GPR30 binds only to estradiol and does not interact with estrone, estriol, progesterone, testosterone, or cortisol. Overexpression of GPR30 has been detected in the hypothalamus, pituitary gland, adrenal glands, kidneys, developing ovarian follicles, lung, heart, and lymphoid tissues [29]. Moreover, nuclear GPR30 overexpression could predict prognosis in women diagnosed with various gynecological malignancies (endometrial, ovarian, and cervical neoplasms), and breast cancer [30–33].

Our aim was to investigate the localization and expression profile of four selected proteins (ARID1A, *NID2*, LRH-1, and GPR30) in different histological subtypes of ECs.

2. Material and method

2.1 Patients and samples

The research was undertaken based on an immunohistochemical analysis of pathological archive slides stored at the Department of Pathology, Princess Anna Mazowiecka Hospital, Warsaw, Poland. Paraffin-embedded tissues were collected from women diagnosed with abnormal vaginal bleeding, or from patients diagnosed with abnormal endometrial thickness during ultrasound examination, and underwent surgery for primary EC, between 2015 and 2020. The tissues were placed on glass slides.

All the tumors were diagnosed at early (I and II) clinical stages of the disease based on the revised FIGO classification [34]. The study comprised 60 neoplasms (based on the WHO staging system): 20 EEC, 20 serous (SER), and 20 CC carcinomas [35]. As a control group, eight normal endometrial samples (NE) were collected from the pathological archives for women who underwent surgery due to benign genital tract disorders (uterine prolapse, uterine leiomyoma). Five normal samples were atrophic, two showed a proliferative features and one displayed secretory feature. Hematoxylin/eosin stained slides were carefully re-examined by highly experienced pathologists (BG and TI) to confirm the diagnosis. No chemotherapy, hormonal therapy, or radiotherapy was administrated to the patients before surgery. The study was approved by the Ethics Committee of the Warsaw Medical University, Warsaw, Poland (KB reference number 43/11).

2.2 Immunohistochemistry

IHC was performed by applying a two-step method as described previously [36, 37]. Briefly, slides were deparaffinized and rehydrated using a routine method. Antigen retrieval by microwave and blockade of endogenous peroxidase activity was performed before adding the primary antibody. The primary, monoclonal, antibodies against ARID1A, *NID2*, LRH-1 (Merck Millipore, Massachusetts, USA) and polyclonal antibody against GPR30 (Abcam, Cambridge, UK), diluted 1:200, were incubated overnight at 4 °C. Afterward, the slides were incubated for 30 min at room temperature with a biotin-free horseradish peroxidase (HRP) enzyme-labeled polymer of EnVision^{+} detection system (DAKO, Denmark). Following reaction with 3,3'diaminobenzidine, the slides were counterstained with hematoxylin, dehydrated, and covered with a coverslip. As positive controls, the normal liver tissue for LRH-1 and normal placenta for other antibodies were used. As negative controls, the slides were stained with normal serum replacing the primary antibody.

2.3 Assessment of immunostaining

Immunostained sections were evaluated under a light microscope (Model SE, Nikon, Japan) at 400× magnification. Based on the staining intensity, the slides were scored as follows: (–) no cells stained; (+) majority of the cells showed weak staining; (++) moderate staining in majority of the cells; (+++) intense staining in majority of the cells. Positive reaction was defined as staining intensity of (+) or above. Two highly experienced pathologists (BG and TI) independently scored the slides without previous knowledge of the clinical and pathological data. Discordant results were repeatedly reviewed and scored based on consensus data.

2.4 Statistical analysis

The Pearson's chi-squared test was applied to determine the IHC differences in four proteins between various histological subtypes of ECs. All of the analyses were conducted using the SPSS version 19.0 (SPSS Inc., Chicago, IL, USA), and a p-value less than 0.05 was considered statistically significant.

3. Results

3.1 Clinico-pathological features

The mean age of the patients was 62 years (range, 43–83 years). There were five (8%) premenopausal women, while 92% (n = 55) were post menopause. Tumor histological grades 2 and 3 were only analyzed. There were 33 (55%) moderately-differentiated and 27 (45%) poorly-differentiated ECs. Patients with early clinical stages (I–II) of the disease were selected. Most of the women were diagnosed at the I (n = 41; 68%) FIGO stage of the disease, while 19 (32%) were stage II. Invasion less than half of the myometrial wall was detected in 35 (58%) ECs; 25 (42%) neoplasms showed invasion above half of the myometrial wall. LVSI and cervical invasion were reported in 16 (26%) and 23 (38%) ECs, respectively.

3.2 ARID1A staining

The ARID1A protein showed both cytoplasmic and nuclear expression (Fig. 1A–D). Altogether, 95% (19 out of 20) and 100% (20 out of 20) of the endometrioid and serous ECs showed intense ARID1A staining, respectively. Interestingly, only four out of 20 (20%) CC carcinomas displayed

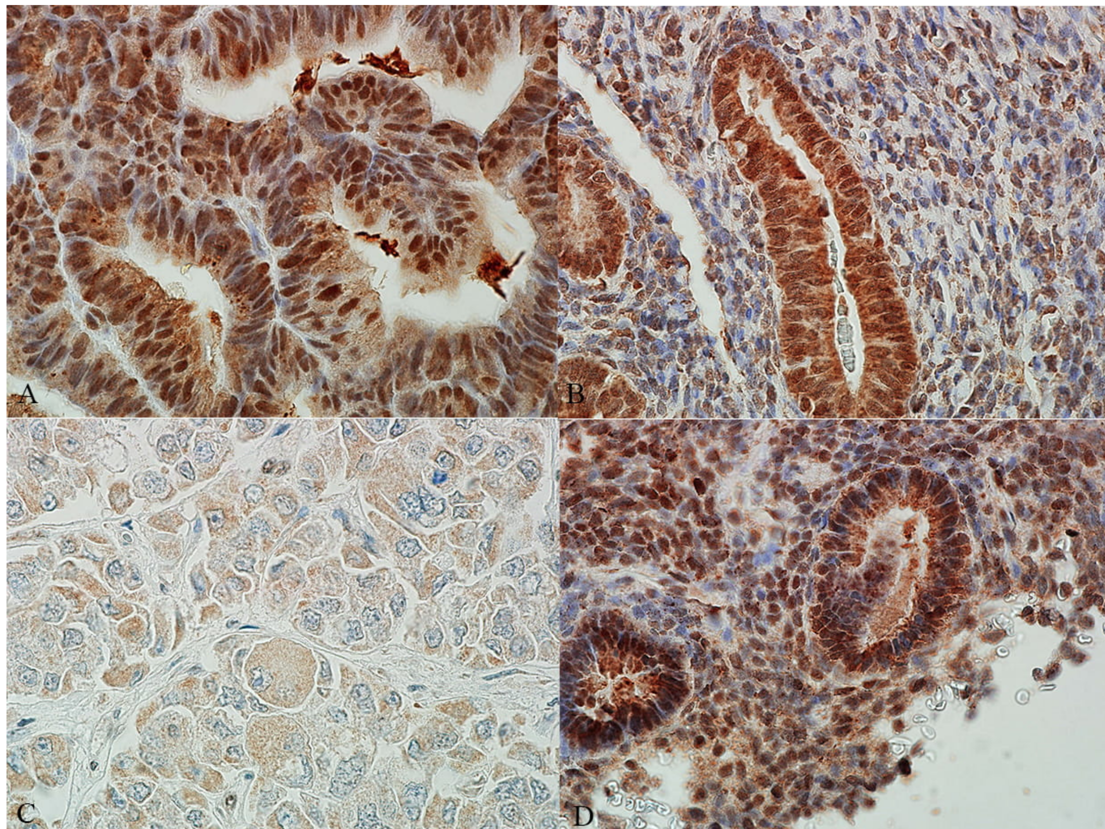

Fig. 1. Immunostaining for ARID1A in ECs and NE. (A) Anti-ARID1A staining in EEC. Glandular and stromal cells show nuclear and cytoplasmic expression (+++) (×200). (B) Anti-ARID1A staining in SER. Nuclear and cytoplasmic expression in glandular and stromal cells (++) (×200). (C) Weak (+) ARID1A cytoplasmic expression in CC carcinoma (×400). (D) Intense nuclear and cytoplasmic ARID1A expression in glandular and stromal cells in normal endometrium with signs of atrophy (×200).

moderate (++) immunoreactivity, while others were only weakly (+) positive. Additionally, all the control endometrial samples showed intense anti-ARID1A staining. A significant difference in ARID1A expression was noted between different histological EC subtypes (CC vs EEC, $p < 0.001$, and CC vs SER, $p < 0.001$). No difference of ARID1A immunoreactivity in relation to tumor grading (G2 vs G3) was reported ($p > 0.05$).

3.3 NID2 staining

Cytoplasmic staining for *NID2* was reported (Fig. 2A–D). In particular, 17 out of 20 (85%) CC carcinomas showed strong immunoreactivity, while 3 out of 20 (15%) revealed weak staining. In all the EER and SER, moderate (++) reaction was reported. Moreover, all the normal endometria were only weakly positive (Fig. 2D).

3.4 LRH-1 staining

Immunostaining with anti-LRH-1 antibody was cytoplasmic (Fig. 3A–D). Most of the endometrioid (19/20; 95%) and serous (19/20, 95%) ECs showed intense LRH-1 staining, which was predominantly detected within the glandular cells. All the CC carcinomas were weakly positive, similar to the normal endometrial slides.

3.5 GPR30 staining

In the case of GPR30, cytoplasmic staining was observed (Fig. 4A–D). Anti-GPR30 immunoreactivity was detected (in glandular and stromal cells) in all the EEC samples, whereas 4 and 16 cases showed weak and moderate staining, respectively. Interestingly, immunohistochemical reactivity of GPR30 was not found in all type II (non-endometrioid) uterine neoplasms and normal endometrial samples. Differences in GPR30 immunoreactivity between EER and SER as well as between EER and CC reached high significant values ($p < 0.001$). The difference between SER and CC carcinomas was not significant ($p > 0.05$).

Table 1 summarized the IHC protein immunoreactivity in normal endometria and the different histological EC subtypes. Clinico-pathological features of EC patients in relation to protein expression patterns are presented in Table 2.

4. Discussion

In the present study, we focus on identifying the localization and staining pattern of selected proteins in different histological subtypes of ECs. Interestingly, most of the EER and SER showed intense ARID1A staining, while only 20% of CC revealed moderate (++) immuno-reactivity. Furthermore, intense nuclear and cytoplasmic staining was detected for anti-ARID1A antibodies in all control slides. Our data

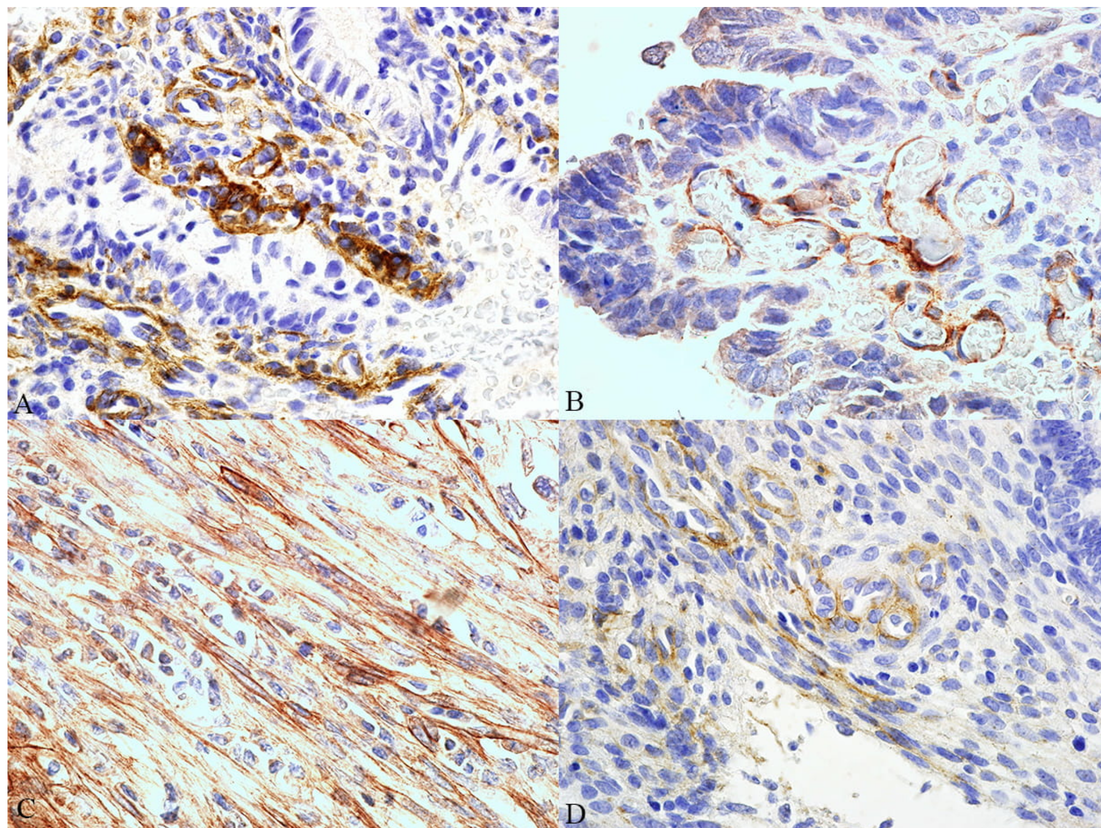

Fig. 2. Immunostaining for *NID2* in ECs and NE. (A) Endometrioid-type EC. Positive anti-*NID2* mesh-like staining in the elements of the intracellular matrix (++) (×100). (B) Anti-*NID2* staining in the SER. Moderately anti-*NID2* mesh-like staining in the elements of the intracellular matrix (++) (×100). (C) Anti-*NID2* staining in the CC carcinoma (×100). (D) Anti-*NID2* staining in the normal endometrium with secretory features. Weak (+) stromal reaction in the elements of extracellular matrix (×100).

Table 1. Summary of the immunohistochemical staining results in normal endometrium and different EC histological subtypes.

Characteristic	Normal endometrium				Endometrioid carcinoma				Serous carcinoma				Clear-cell carcinoma			
	– (%)	+ (%)	++ (%)	+++ (%)	– (%)	+ (%)	++ (%)	+++ (%)	– (%)	+ (%)	++ (%)	+++ (%)	– (%)	+ (%)	++ (%)	+++ (%)
ARID1A				8 (100)			1 (5)	19 (95)				20 (100)		16 (80)	4 (20)	
NID2		8 (100)					20 (100)				20 (100)			3 (15)		17 (85)
LRH-1		8 (100)				1 (5)		19 (95)		1 (5)		19 (95)		20 (100)		
GPR30	8 (100)					4 (20)	16 (80)		20 (100)				20 (100)			

support the view that weak ARID1A expression may be associated with gene alterations in selected histological subtypes of EC. These results are consistent with those of Heckl *et al.* [38], where ARID1A expression was found to be significantly ($p < 0.001$) related to EC histological subtypes. Positive nuclear staining was observed more frequently in the EEC, whereas it was uncommon in CCs [38]. They finally suggested that "reduced ARID1A expression is mostly induced by nonsense mutations as well as insertions and deletions in the gene-coding region". Furthermore, a lack of ARID1A staining was a predictor for unfavorable prognosis in women affected by EC [10]. Thus, ARID1A staining patterns may serve as a prognostic indicator, especially in women affected by endometrial carcinomas of CC histology as well as in EC of advanced clinical stage [11].

There are only limited data investigating the expression patterns of *NID2* and LRH-1 in different EC histological subtypes (PubMed©) [5, 25]. Nevertheless, the present study identified cytoplasmic *NID2* staining in most of the cancers and control endometria samples. However, only three CC carcinomas revealed weak *NID2* reactivity. A lack of/weak nidogen 2 staining may be connected with various mechanisms of gene silencing, for example, *NID2* methylation [16]. Furthermore, "loss of nidogen expression may favor invasion and metastasis of cancer cells by loosening cell interaction with the basal membrane and by weakening the strength of the basement membrane itself" [5]. Lack of *NID2* may also facilitate the passage of tumor cells through the basement membrane, leading to increased cancer metastatic potential [16–18]. Moreover, methylation of *NID2* reduces its expression levels and promotes the development of lung cancer [39].

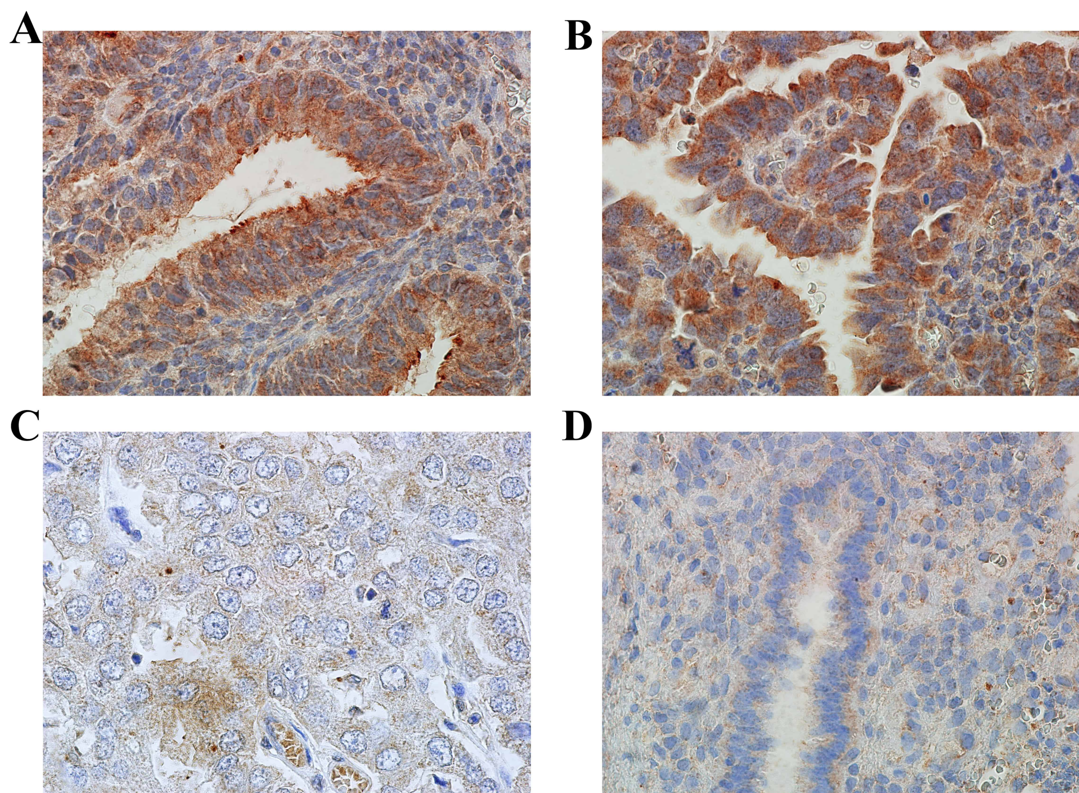

Fig. 3. Immunostaining for LRH-1 in ECs and NE. (A) Intense (+++) nuclear and cytoplasmic LRH-1 expression in the glandular cells and weak (+) in stromal cells in EEC ($\times$200). (B) Intense (+++) cytoplasmic LRH-1 expression in the glandular cells and weak (+) cytoplasmic expression in stromal cells in SER ($\times$200). (C) Weak (+) cytoplasmic LRH-1 expression in CC carcinomas ($\times$400). (D) Normal endometrium with inactive glands. Weak (+) cytoplasmic LRH-1 staining in the glandular and stromal cells ($\times$200).

Additionally, the loss of *NID2* also has a pathogenetic role in the ovarian, esophageal, and hepatocellular tumorigeneses [19, 40, 41].

EC cells express LRH-1 at the mRNA and protein levels and activate the genes encoding the steroidogenic enzymes involved in estradiol synthesis [25]. The LRH-1 is a well-known regulator of the steroidogenic gene expression pattern in the normal gonadal and adrenal cells [25]. LRH-1 promotes malignant transformation, and protein expression has been well documented in several types of human malignancies [24, 25]. LRH-1 was found to be overexpressed in the non-small cell lung cancer tissues, being correlated with poorer differentiation (p = 0.023), pathological tumor classification ($p < 0.001$), advanced pathological stage (p = 0.017), histological subtype (p = 0.031), and positive lymph node metastases ($p < 0.001$) [24]. In colon cancer, the OS of patients with positive LRH-1 expression was significantly lower than in those with negative expression [22]. Moreover, LRH-1 was also overexpressed in pancreatic carcinoma and was associated with increased metastatic potential [20, 23].

In our study, intense cytoplasmic LRH-1 staining was observed in the glandular epithelium in EEC and SER, whereas weak reaction was detected in the CCs and normal endometria. Previously, enhanced proliferation of endometrial cells was associated with the transcriptional cooperation of steroidogenic factor 1 (SF1) and LRH-1 with the members of the AP-1 family [25].

GPR30, an alternative intracellular estrogen receptor, regulates a number of important biological functions [27, 42]. Additionally, pharmacological inhibition of GPR30 activity prevents estrogen-mediated tumor growth *in vivo* [43]. Down-regulation of GPR30 reduced growth and invasion of cells treated with 17β-estradiol. GPR30 mediates the rapid non-genomic effects of estrogen and is a highly specific receptor for the 17β-estradiol [44]. GPR30 is overexpressed in carcinoma of the uterine cervix, endometrium, ovary, and also in breast cancer after tamoxifen treatment [30–32, 45–50]. Interestingly, although immunohistochemistry showed diffuse GPR30 nuclear staining, intracytoplasmatic or membrane staining was observed in some ovarian cancers from Korean women [50]. Moreover, nuclear, but not cytoplasmic, expression of GPR30 predicts unfavorable OS and DFS in women who have suffered from gynecological malignancies [30, 31].

In the current research, GPR30 was cytoplasmatically expressed in the EECs, but it was absent in all non-endometrioid uterine tumors. Immunostaining of GPR30 could distinguish type I from type II ECs, although a few EECs also lacked GPR30 immunoreactivity. Interestingly, a cytoplasmic GPR30 staining characterizes a subgroup of endometrioid-subtype ECa. In contrast, a previous study

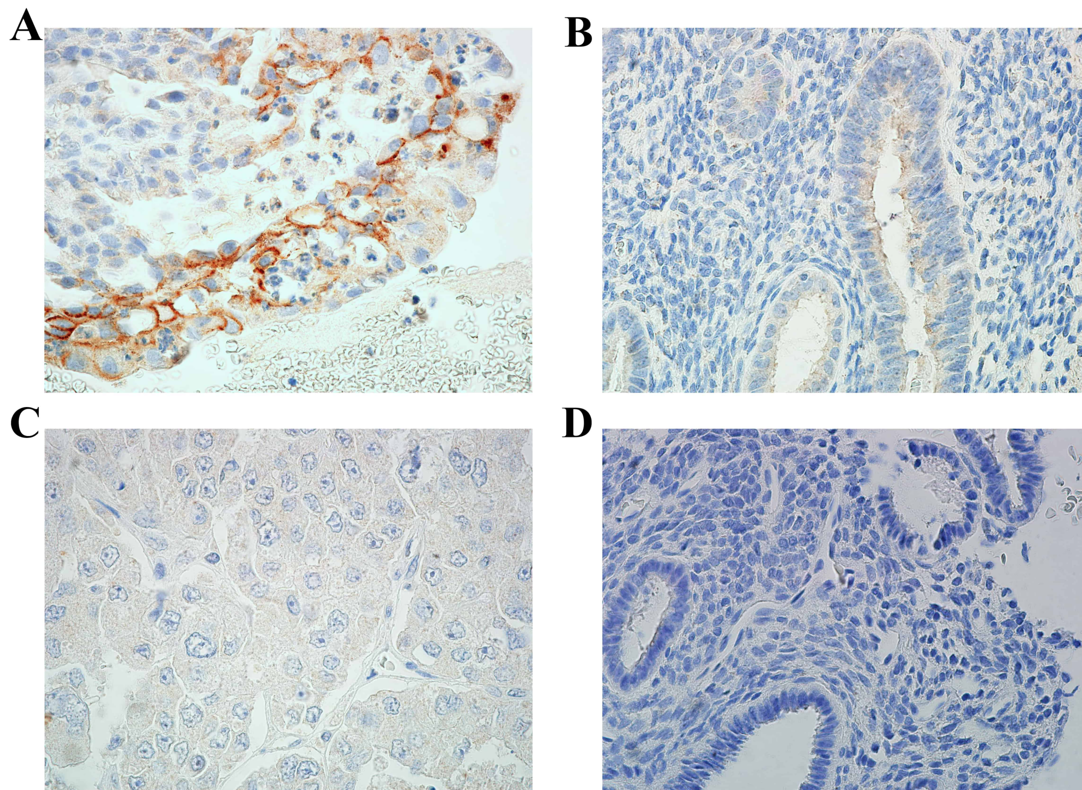

Fig. 4. Immunostaining for GPR30 in ECs and NE. (A) Cytoplasmic reaction of GPR30 in glands and stroma with intensification in cell membranes in EEC (×200). (B) Negative anti-GPR30 cytoplasmic reaction in glandular cells in SER (×200). (C) Negative anti-GPR30 cytoplasmic reaction in glandular cells in CC carcinoma (×400). (D) Normal endometrium with sign of atrophy. Negative anti-GPR30 staining (×200).

Table 2. Clinico-pathological features of sixty EC patients in relations to proteins expression pattern.

Characteristic	n	ARID1A positive	NID-2 positive	LRH-1 positive	GPR-30 positive
		n (%)*	n (%)*	n (%)*	n (%)*
Patient age (years)					
<50	4	4 (100)	4 (100)	4 (100)	2 (50)
50–60	12	12 (100)	12 (100)	12 (100)	3 (25)
>60	44	44 (100)	44 (100)	44 (100)	15 (34)
Menopausal status					
premenopausal	5	5 (100)	5 (100)	5 (100)	4 (80)
postmenopausal	55	55 (100)	55 (100)	55 (100)	16 (29)
Histological grade					
G2	33	33 (100)	33 (100)	33 (100)	7 (21)
G3	27	27 (100)	27 (100)	27 (100)	13 (48)
FIGO					
I	41	41 (100)	41 (100)	41 (100)	11 (27)
II	19	19 (100)	19 (100)	19 (100)	9 (47)
Myometrial invasion					
<50%	35	35 (100)	35 (100)	35 (100)	10 (29)
>50%	25	25 (100)	25 (100)	25 (100)	10 (40)
LVSI					
present	16	16 (100)	16 (100)	16 (100)	7 (44)
absent	44	44 (100)	44 (100)	44 (100)	13 (30)
Cervical invasion					
present	23	23 (100)	23 (100)	23 (100)	5 (22)
absent	37	37 (100)	37 (100)	37 (100)	15 (41)

*Positive reaction was defined as staining intensity of (+) or above.

presented no difference in the positivity and intensity of nuclear GPR30 immunostaining between the EC subtypes in the Chinese population [49]. They also reported that nuclear GPR30 positivity was not associated with menopausal status or ER-reactivity [49]. However, it should be emphasized that as high as 34% of EC women were premenopausal. Presently, cytoplasmic, but not nuclear, expression of GPR30 was reported, and most of our patients (92%) were postmenopausal. Therefore, endometrial GPR30 expression may not be associated with population diversity but may be connected with women's hormonal status. Interestingly, "the overall positivity of GPR30 in endometrial cancer was 87% in Caucasians which was higher than Chinese population" [30].

Our study showed a few limitations. Firstly, although our study group was carefully matched from one Institution, the number of patients was limited. Secondly, early-staged (I–II due to FIGO) ECs with moderately- and poorly-differentiated histology were only investigated. Thirdly, we performed IHC staining, whereas the molecular mechanisms responsible for a lack of ARID1A or GPR30 expression remained unresolved. That is why we started the experiments searching for the molecular mechanisms responsible for the altered ARID1A or GPR30 expression in different histological subtypes of EC.

5. Conclusions

Weak ARID1A expression may be associated with gene alterations in selected EC histological subtypes. GPR30 staining may help to differentiate various histological EC subtypes.

Abbreviations

DFS, disease-free survival; CC, clear-cell carcinoma; EC, endometrial cancer; EEC, endometrioid endometrial carcinoma; IHC, immunohistochemistry; LVSI, lymphovascular space invasion; MI, myometrial invasion; NE, normal endometrium; OS overall survival; SER, serous carcinoma.

Author contributions

WS—Project development, Data collection, Manuscript writing. OS—Project development, Data collection; KC—Data analysis; BG—Project development, Data analysis; Manuscript writing; TI—Data analysis; AS—Project development, Manuscript writing. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Our study is compliant with the guidelines for human studies and research was conducted ethically based on the World Medical Association Declaration of Helsinki. The requirement for informed consent was waived due to the retrospective nature of the research.

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Conflict of interest

The authors declare no conflict of interest.

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